

Studies on the respiratory system of
Campylobacter mucosalis.

Celia Frances Goodhew.

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Faculty of Veterinary Medicine.

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Declaration

In accordance with the regulations of the University of Edinburgh, the work presented in this thesis, apart from the acknowledged assistance is my own. Part of the work has been published and these papers appear in the appendix.

Celia F. Goodhew.

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Abstract

The microaerophilic, catalase-negative, gram-negative bacterium Campylobacter mucosalis was grown microaerobically with either hydrogen or formate as electron donor. A model is proposed for the respiratory adaptation of the organism to falling oxygen tensions during growth. During the early stages of growth, when the oxygen concentration is relatively high, the oxidation of formate is a two-stage branched process involving hydrogen peroxide production and its peroxidative removal. In later stages of growth, when the oxygen concentration is lower as a result of cellular respiration, the principal electron flow is linear to a membrane-bound cytochrome-c oxidase which reduces oxygen directly to water. The reactivity of cytochrome c-553 with the soluble peroxidase and the membrane-bound cytochrome-c oxidase was consistent with this cytochrome operating as physiological electron donor to both enzyme systems.

The cytochrome-c peroxidase was partially purified and some of its properties determined. The cytochrome-c oxidase and formate oxidase were both membrane-bound and exhibited a high affinity for oxygen.

Hydrogen peroxide was produced during the oxidation of formate, and accumulated when electron flow to the cytochrome-c peroxidase was inhibited. The capacity for hydrogen peroxide production was shown to be higher in young cells. The levels of cytochrome c-553 and of the peroxidase were higher in cells harvested earlier in growth. In cells harvested later in growth, the levels of cytochrome-c oxidase increased and the levels of c-553 decreased.

A proton gradient with lower external pH was developed with either hydrogen or formate as electron donor, and either oxygen or hydrogen peroxide as the terminal electron acceptor. A respiratory role for hydrogen peroxide in this catalase-negative organism was proposed and discussed in relation to its microaerophilic nature.

Haem-stained SDS-PAGE gels was one of the methods used to

quantify cytochrome c-553. This method was shown to be generally applicable to the quantification of c-type cytochromes and to be useful during investigations into the induction and location of bacterial cytochromes-c.

Abbreviations

ABTS	2,2'-azino-bis 3-ethylbenzthiazoline sulfonic acid
AP	Alkaline phosphatase
ATCC	American type culture collection
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CBA	Columbia blood agar
CCCP	Carbonyl cyanide m-chlorophenyl-hydrazone
CCP	Cytochrome-c peroxidase
DCPIP	2,6-dichlorophenol indophenol
DEAE	Diethylaminoethyl
d.f.	Degrees of freedom
DMB	3,3'-dimethoxy benzidine
DMPD	N,N-dimethyl-p-phenylenediamine
DNA	Deoxyribonucleic acid
DNA'ase	Deoxyribonuclease
d.o.t.	Dissolved oxygen tension
DTT	Dithiothreitol
e ⁻	Electron
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycolbis-(aminoethylether)tetraacetic acid
EM	Electron microscopy
ETS	Electron transport system
FAD	Flavin adenine dinucleotide
FBP	Ferrous sulphate/sodium bisulphite/sodium pyruvate
FDH	Formate dehydrogenase
FITC	Fluorescein isothiocyanate
FR	Fumarate reductase
Hb	Haemoglobin
HEPES	N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid
HHC	Horse heart cytochrome-c
HOQNO	2-heptyl 4-hydroxyquinoline-N-oxide
Hp	Haptoglobin
ICDH	Isocitrate dehydrogenase
INT	Iodonitrotetrazolium

LDS	Lithium dodecyl sulphate	
LPS	Lipopolysaccharide	
MDH	Malate dehydrogenase	
MOPS	3-(N-morpholino) propanesulfonic acid	-
M _r	Molecular weight	
NAD	Nicotinamide adenine dinucleotide	
NADH	" " "	reduced
NADP	" " "	phosphate
NADPH	" " "	" reduced
NAG	N-acetyl glucosamine	
NAM	N-acetyl muramic acid	
NBT	Nitroblue tetrazolium	
NCTC	National collection of type cultures	
NEM	N-ethylmaleimide	
PAGE	Polyacrylamide gel electrophoresis	
PBS	Phosphate buffered saline	
Pi	Phosphate	
PMS	Phenazine methosulphate	
p.s.i	Pounds per square inch	
Q	Ubiquinone	
RDSVS	Royal (Dick) School of Veterinary Studies	
SDS	Sodium dodecyl sulphate	
SKH	Sucrose/KCl/Hepes	
SOD	Superoxide dismutase	
STV	Saline trypsin versene	
TMBZ	3,3',5,5'-tetramethylbenzidine	
TMPD	N,N,N',N'-tetramethyl p-phenyldiamine	
TRIS	Tris(hydroxymethyl)amino methane	
TTC	Triphenyl tetrazolium chloride	
UV	Ultraviolet	
YCCP	Yeast cytochrome-c peroxidase	

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Chapter 1

Introduction

This study is concerned with the microaerophilic respiration of C.mucosalis. The following sections will review

- 1) The similarities between the respiratory systems of bacteria and mitochondria.
- 2) The morphology and ecology of Campylobacter species.
- 3) The current knowledge on the terminal aerobic respiratory systems in microaerophilic Campylobacter species.
- 4) The possible causes of oxygen toxicity in microaerophiles.

A proposed model for the microaerophilic respiration of C.mucosalis will then be presented, which was derived as a result of the present investigations.

1) Bacterial and Mitochondrial respiratory systems.

Energy conservation in bacteria by the formation of adenosine 5'-triphosphate (ATP) can occur by substrate level phosphorylation and by oxidative or photophosphorylation. The small number of substrate level phosphorylations that have been identified are catalysed by soluble cytoplasmic enzymes (Haddock and Jones 1977). Most of the reactions involved in ATP synthesis are coupled to electron transport reactions which are driven by light (in phototrophs) or by the oxidation of low potential organic or inorganic compounds linked to the reduction of high potential electron (e^-) acceptors (Haddock and Jones 1977).

The electron transport chains of bacteria are branched, reflecting the diversity of substrates and e^- acceptors utilised, but they have many features in common with the mitochondrial electron transport system (E.T.S.). The following diagram (Figure 1a) and text will outline the general similarities of the eukaryote and prokaryote

respiratory systems, and the diversity of the latter.

Electron Transport Systems

Mitochondrial

Bacterial

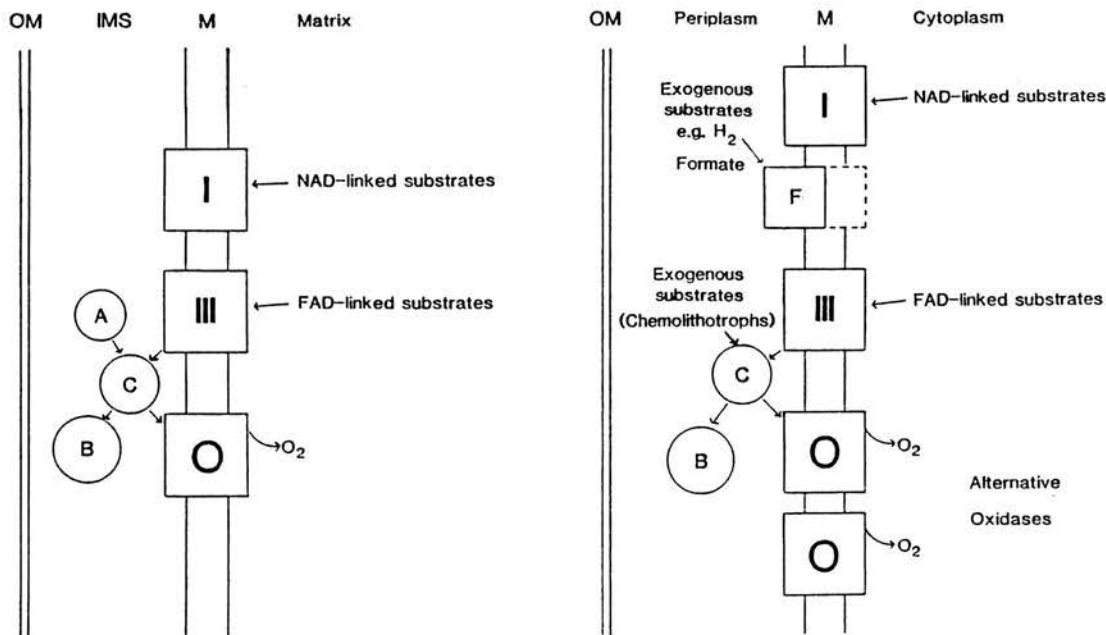


Figure 1a

Outer membrane O.M., Intermembrane space I.M.S., Membrane M., Cytochrome-c C., Intermembrane enzyme (e.g. sulphite oxidase) donating e^- to cytochrome-c A., Intermembrane or periplasmic enzyme (e.g. cytochrome-c peroxidase in yeast and bacteria) accepting e^- from cytochrome-c B., Formate dehydrogenase or hydrogenase F., Oxidase O., Direction of e^- transfer -->

Apart from rare exceptions (the cytochrome c peroxidase and sulphite oxidase shown in the diagram) the mitochondrial respiratory chain involves linear e^- transfer from complex III (the ubiquinol-cytochrome c oxidoreductase) via cytochrome c to the terminal oxidase (complex IV, cytochrome aa_3).

Bacterial respiratory chains are more diverse and therefore the general diagram cannot cover all the possibilities. However the common features of bacterial and mitochondrial respiratory chains can be illustrated with reference to figure 1a.

Mitochondrial and bacterial systems obtain e^- from nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) linked oxidation of substrates in the mitochondrial matrix. In addition many bacteria can directly oxidise exogenous substrates, such as hydrogen and formate, using specialised enzymes. The diagram shows these enzymes (hydrogenase and formate dehydrogenase) to be situated on the periplasmic face of the cytoplasmic membrane, but this location has not been conclusively resolved. The formate dehydrogenase (FDH) and hydrogenase in C.jejuni were reported to be facing the periplasm (Hoffman and Goodman 1982). The FDH of Wolinella succinogenes has been so located by the similar activities of the enzyme in intact and lysed cells when reacting with dyes that do not permeate through the cytoplasmic membrane (Kroger 1978). The FDH of E.coli was reported to be facing the cytoplasm (Garland et al. 1975) based on the stoichiometry of H^+ translocation, but there was some doubt as to the validity of the result due to the pH changes resulting from the formation of CO_2 during the oxidation of formate. The enzyme may however be transmembranous (Van der Plas et al. 1983, Ingledew and Poole 1984) although the latter authors do not reject the possibility of a periplasmic location for the active site.

A soluble hydrogenase of Desulfovibrio was located in the periplasm (Odom and Peck 1981b and 1984, Bell et al. 1974) and donates e^- to the electron transport system via cytochrome c_3

(Odom and Peck 1981a). Uncertainty exists for the location of membrane-bound hydrogenases, which were reported to be cytoplasmic facing (Van der Plas et al. 1983) or transmembranous (Graham 1981). In general, hydrogenases that oxidise H_2 for energy production (compared to NAD^+ reduction for biosynthetic purposes) were reported to be extracytoplasmic (Hooper and DiSpirito 1985). Regardless of the precise orientation of these enzymes, they supply e^- to the electron transport system at the level of Q (ubiquinone).

Mitochondrial and bacterial respiratory chains both involve complexes containing ubiquinone (Q) or a variant of Q such as desmethylmenaquinone or menaquinone (vitamin K) which is found in E.coli (Polglase et al. 1965) and W.succinogenes (Kroger et al. 1979). The site of action of the quinol analogue and respiratory inhibitor 2-n-Heptyl 4-hydroxyquinoline-N-oxide (HOQNO) is proposed to be between cytochrome b and c_1 (Van Ark et al. 1981).

Electrons from the $b-c_1$ complex (complex III) are transferred (as are e^- from the oxidation of exogenous substrates by chemolithotrophic bacteria, and e^- from intermembrane oxidation of sulphite and lactate in some mitochondrial systems) to cytochrome c. The cytochrome c acts as a diffusible mediator between reductases (both soluble and membrane-bound) and the oxidases (which can also be soluble or membrane-bound). The mitochondrial and bacterial c-type cytochromes that have this function are all of the class I type (Pettigrew and Moore 1987). These cytochromes are characterised by their high potential, the methionine-histidine ligation of the iron and by structural homology. Class I c-type cytochromes therefore hold a key role as a branch point for e^- transfer to alternative terminal oxidising enzymes.

The mitochondrial membrane-bound oxidase (complex IV) is an aa_3 type cytochrome, whereas bacteria have multiple oxidases which can be induced in response to growth conditions. Oxidase types found in bacteria and mitochondria and their spectral characteristics are described in chapter 4

section 1.

Soluble oxidising enzymes are rare in mitochondrial systems, an example being the cytochrome-c peroxidase in yeast that accepts e^- from cytochrome c (Yonetani and Ray 1965, 1966). Soluble oxidising enzymes of bacteria include the denitrifying enzymes and also the peroxidases. The best studied example of the bacterial peroxidases is the Pseudomonas aeruginosa cytochrome-c peroxidase (Ellfolk and Soininen 1970, 1971, Ellfolk et al. 1983, 1984, Soininen and Ellfolk 1972, 1973, 1975, Soininen et al. 1970, 1973) which oxidises a periplasmic c-type cytochrome. The H_2O_2 removed by the action of cytochrome c peroxidase is probably important for the detoxification of this reactive species, which is formed from the partial reduction of oxygen (Halliwell and Gutteridge 1985, Gutteridge et al. 1985). The rate of formation and removal of reactive species such as superoxide and peroxide is thought to explain the lack of aerotolerance exhibited by microaerophilic bacteria such as Campylobacter mucosalis. The causes of microaerophily will be discussed in section 4 of this chapter.

2) Morphology and ecology of Campylobacter species.

The genus Campylobacter belongs to the Spirillaceae family (Krieg and Smibert 1974) which also includes the genus Spirillum. Members of the Campylobacter family are small, curved or spiral rods with a single polar flagellum (Smibert 1974, 1978). Most are parasitic (Krieg and Hoffman 1986) including pathogens of man and animals (Smibert 1974). They are found in the reproductive organs, intestinal tract and oral cavity (Smibert 1974). Some Campylobacters are saprophytic (Smibert 1978), one species (C.nitrofigilis) is a marine N_2 -fixing species (Krieg and Hoffman 1986). C.mucosalis (Roop et al. 1985) previously called C.sputorum subspecies mucosalis (Lawson et al. 1981), was originally isolated from lesions of porcine intestinal adenomatosis (Lawson and Rowland 1974). Intracellular organisms have been shown to undergo division while located within the cytoplasm of adenomatous

epithelial cells (Lawson et al. 1976). The high recovery of C.mucosalis from adenomatous lesions, and the low recovery of organisms from faeces (Lawson et al. 1976) suggests that the intracellular location is the normal habitat for the bacterium.

3) Terminal respiration in microaerophilic Campylobacter species.

Campylobacter species are unable to use carbohydrates, which are neither fermented nor oxidised (Smibert 1974). They obtain energy by oxidative phosphorylation using tricarboxylic acid cycle (TCA) intermediates as substrates (Smibert 1978). Hydrogen and formate can also operate as e^- donor to C.mucosalis (Lawson et al. 1981), C.fetus (formally C.fetus subspecies intestinalis) (Harvey and Lascelles 1980), C.sputorum subspecies bubulus (Niekus et al. 1980b) and C.jejuni (formally C.fetus subspecies jejuni) (Carlone and Lascelles 1982) with O_2 , at reduced oxygen tensions, as e^- acceptor. [Most of these species can also grow anaerobically with fumarate as e^- acceptor except for C.jejuni (Lascelles and Calder 1985) which has the necessary enzymes (formate dehydrogenase and fumarate reductase) but none the less has a requirement for O_2].

The reduction of cytochromes c and cytochromes b by H_2 and formate and the presence of cytochrome-c that was reducible by ascorbate plus tetramethyl-phenylene diamine (TMPD), indicated that the e^- transfer from H_2 or formate to the oxidase involves the mediation of high potential c-type cytochromes in C.jejuni (Carlone and Lascelles 1982, Hoffman and Goodman 1982), C.fetus (Lascelles and Calder 1985, Harvey and Lascelles 1980) and C.sputorum ss. bubulus (Niekus et al. 1980a). A high potential, small cytochrome c that was probably basic (as it did not bind to the anion exchanger DEAE-Sephacel at pH 8.5) was partially purified from C.fetus (Lascelles and Calder 1985), but its function in the respiratory chain was not determined. A soluble, high potential possibly acidic cytochrome c was purified from C.jejuni (Hoffman and Goodman

1982) with an α -peak maximum of 554nm, but was otherwise uncharacterised. All of the c-type cytochromes in C.mucosalis were reducible by formate or hydrogen but the presence of a high potential (ascorbate reducible) c-type was not originally detected (Lawson et al. 1981). A high potential soluble c-type cytochrome was later identified in C.mucosalis by potentiometric titrations of crude extracts (Elkurdi et al. 1982). This c-type cytochrome (c-553) was isolated and its redox potential, amino acid composition and spectroscopic properties determined (Goodhew et al. 1988). Cytochrome c-553 was shown to be typical of the class I c-types, although it is one of the few basic bacterial cytochromes (Goodhew et al. 1988).

Cytochrome oxidases of the a-, o- or d-type were not conclusively identified by spectral analysis in C.sputorum ss. bubulus (Niekus et al. 1980a, 1980b), C.jejuni (Hoffman and Goodman 1982) or C.fetus (Harvey and Lascelles 1980, Lascelles and Calder 1985). The branched nature of the Campylobacter terminal oxidase enzymes was demonstrated by the presence of two or more oxidase systems in C.jejuni (Hoffman and Goodman 1982), C.fetus (Harvey and Lascelles 1980) and C.sputorum ss. bubulus (Niekus et al. 1980a, 1980b, Niekus and Stouthamer 1981). Formate oxidation in C.sputorum ss. bubulus occurred via two pathways of e^- transfer (Niekus et al. 1980a). One of the pathways had a high affinity for O_2 and was inhibited by HOQNO, antimycin A (Niekus et al. 1980a) and glutaraldehyde (Niekus and Stouthamer 1981) and thus involved the membrane-associated respiratory chain. The other path was of low O_2 affinity and did not involve the respiratory chain (Niekus et al. 1980a) but was also located on the cytoplasmic membrane by a cytochemical staining technique (Niekus et al. 1980c). The low affinity system produced H_2O_2 (implicated as a cause of oxygen toxicity, see section 4 of this chapter) which was removed by the action of a cytochrome c peroxidase (Niekus et al. 1980b). Inhibition of the respiratory chain probably affected the removal of H_2O_2 by restricting the supply of reduced cytochrome c to the peroxidase (Niekus and Stouthamer

1981), as in the presence of the respiratory inhibitor glutaraldehyde the peroxidase activity itself (measured with reduced horse heart cytochrome c as e^- donor) was not inhibited (Niekus et al. 1980b).

Although the activities, oxygen affinities and inhibitor sensitivities of oxidising enzymes in several Campylobacter species have been investigated, the physiological e^- donors to the terminal oxidases or peroxidase have not been determined. This subject will be investigated in C.mucosalis during the current study.

4) Oxygen toxicity during aerobic respiration by microaerophiles.

Microaerophilic organisms including most Campylobacter species use O_2 for energy yielding respiration, but are killed by high levels of oxygen. These organisms are therefore sensitive to toxic forms of oxygen which include superoxide, hydrogen peroxide, hydroxyl radicals and singlet oxygen (Gutteridge et al. 1985, Halliwell and Gutteridge 1985). The microaerophile can tolerate low levels of oxygen when the generation of toxic forms will be correspondingly lower.

Toxic forms of oxygen arise by non-biological means, for example by photochemical generation, and are also produced by the microaerophiles themselves (Krieg and Hoffman 1986). The H_2O_2 produced during the oxidation of formate by C.sputorum ss. bubulus as described in section 3, is an example of the biological formation of a toxic product of oxygen reduction. The level of O_2 that can be tolerated will depend upon several factors (Krieg and Hoffman 1986). These factors include the rate of production of toxic species, the susceptibility of cellular components to damage, the rate of repair to such damaged components, the presence, absence and efficiency of detoxifying enzymes such as superoxide dismutase (SOD), catalase and peroxidase within the organism itself, and the presence, absence and efficiency of growth media supplements that can quench toxic forms of oxygen.

a) Rate of production.

The rate of generation of toxic forms of oxygen that occurs by non-biological means in the dark and to a greater extent in the light will decrease under lowered oxygen tensions. The rate of generation by biological means depends upon the respiratory substrate and the affinity that the oxidising enzyme has for O_2 .

The oxidation of formate and lactate by C.sputorum ss. bubulus generated O_2^- (Niekus et al. 1978) but only the former substrate caused significant H_2O_2 production. Similarly only formate caused H_2O_2 production in C.jejuni (Hoffman et al. 1979a). The H_2O_2 -producing formate oxidase had a low affinity for oxygen (Niekus et al. 1980b) and consequently the rate of peroxide production was enhanced as the O_2 tension was raised. If H_2O_2 production occurred via a high affinity oxidase, then production of the toxic species would be the same over a wide range of oxygen levels.

b) Susceptibility to cellular damage.

Possible targets for damage by toxic species include outer membrane proteins, redox components of the cytoplasmic membrane, enzymes of the periplasmic space and DNA (Krieg and Hoffman 1986). The lactate dehydrogenase (Niekus et al. 1977) and formate dehydrogenase (Niekus et al. 1980a) of C.sputorum ss. bubulus and the formate dehydrogenase of C.jejuni (Carlone and Lascelles 1982) were reported to be susceptible to oxygen damage. Several of the microaerophilic Campylobacter species are known to contain cytochromes of low redox potential and cytochrome oxidases of high affinity for O_2 , both of which may be susceptible to damage by H_2O_2 or O_2^- (Krieg and Hoffman 1986).

c) Rate of repair.

E.coli could rapidly repair DNA breakage caused by H_2O_2 however the lack of DNA repair enzymes in microaerophiles could result in oxygen toxicity (Krieg and Hoffman 1986).

d) Presence of detoxifying enzymes in the bacterium and radical scavengers in the growth medium.

H_2O_2 is destroyed by catalase, peroxidase, MnO_2 , pyruvate, dithiothreitol and charcoal. O_2^- is destroyed by superoxide dismutase (SOD), charcoal (Krieg and Hoffman 1986), and by the combined non-enzymatic reaction of iron and bisulphite (Smibert 1978). The absence of detoxifying enzymes such as SOD, catalase and peroxidase might be expected to correlate with a microaerophilic phenotype. The activity of SOD, which converts O_2^- into H_2O_2 , was induced by C.sputorum ss. bubulus when grown with higher levels of O_2 (Niekus et al. 1978). However, SOD was reported to be present in most microaerophilic organisms (Krieg and Hoffman 1986) and the level of SOD in C.jejuni was not related to aerotolerance (Hoffman et al. 1979b). This suggested to Krieg and Hoffman (1986) that lack of catalase may be more important than SOD in determining the toxic level of O_2 . The catalase negative Campylobacters C.sputorum ss. bubulus and C.mucosalis are indeed microaerophilic, but so are the catalase positive species C.jejuni and C.fetus. However, both of the catalase negative species contain peroxidase and some organisms contain both peroxidase and catalase but are microaerophilic in nature. The lack of protection conferred may be due to the inability of these enzymes to cross membranes resulting in the ineffectiveness of SOD, catalase and peroxidase to destroy exogenous toxic forms of oxygen (Krieg and Hoffman 1986). The enhanced aerotolerance of C.fetus (Smibert 1978, Krieg and Hoffman 1986) and C.jejuni (Hoffman et al. 1979a, Hoffman and Goodman 1982) that occurred when catalase, SOD and the ferrous sulphate/sodium bisulphite/sodium pyruvate (FBP) supplement were added to the media would tend to support this proposal, with the media supplements removing the exogenous toxic species. However, aerotolerance of the catalase negative, peroxidase and SOD positive organisms C.sputorum ss. bubulus and C.mucosalis is not enhanced by the addition of catalase, SOD or FBP to the media (Niekus et al. 1980b, Krieg and Hoffman 1986, Smibert 1978). This anomaly has not been

satisfactorily explained in the literature.

C.mucosalis like C.sputorum ss. bubulus is a catalase negative microaerophile (Lawson et al. 1975). In this thesis will be presented the results of experiments to investigate the microaerobic respiration of C.mucosalis. From these results a model was derived for the respiratory adaptation of C.mucosalis to changes in O_2 concentrations that could explain the microaerophilic nature of this organism. This model will be discussed fully in chapter 7. However to aid the reader during the presentation of the experimental data (chapters 3 to 6), the proposed model is presented below.

The proposed model involves respiratory adaptation to falling O_2 concentrations that occur during growth. During the early stages of growth, when the O_2 concentration is relatively high, the oxidation of formate principally occurs via a two-stage branched process involving the production of H_2O_2 followed by its peroxidative removal. In the later stages of growth, when the oxygen concentration has fallen, the predominant electron flow is linear to a membrane-bound cytochrome-c oxidase which reduces O_2 directly to H_2O .

Model for the microaerobic respiration of *C.mucosalis*.

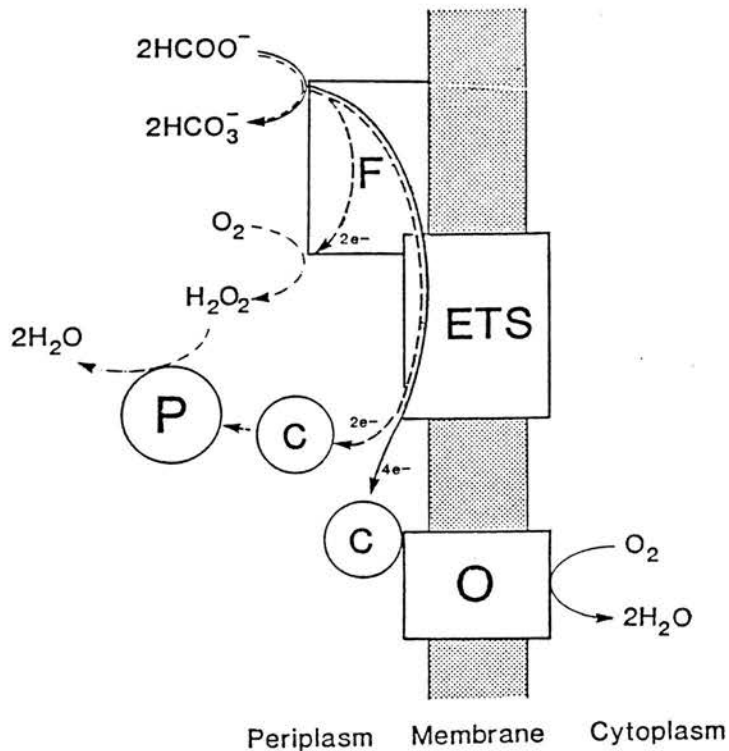


Figure 1b. Alternative pathways of electron flow during respiration by *C.mucosalis*. Two different pathways for the oxidation of formate are shown. The broken line shows the proposed principal flow of electrons occurring in cells harvested early in growth. This involves reduction of O_2 to H_2O_2 by a formate oxidising enzyme (F) followed by reduction of H_2O_2 to H_2O by a cytochrome-c peroxidase (P) that receives electrons from the electron transport system (ETS) via cytochrome c-553 (C). The solid line shows the proposed principal flow of electrons leading to a conventional reduction of O_2 to H_2O by a membrane-bound terminal oxidase (O).

Chapter 2

Materials and Methods

Abbreviations.

mM and uM = millimolar and micromolar respectively.

mmol, umol and pmol = millimoles, micromoles and picomoles respectively.

Growth of bacteria

Campylobacter mucosalis f. C.sputorum subspecies mucosalis (Strain 1248, NCTC 253/72) (Roop et al. 1985) was a gift from G.H.K. Lawson (Veterinary Pathology RDSVS). The organism was grown routinely on plates of Columbia agar base (Oxoid, Basingstoke, U.K.) supplemented with 5% horse serum or whole horse blood (Gibco, Paisley, U.K.) in a microaerobic atmosphere initially comprising 4.5% O₂/5% CO₂/15.5% N₂/75% H₂. This level of O₂ is equivalent to 20% of atmospheric O₂ tensions, and was obtained by partial evacuation of the growth jar (600mm Hg). Higher O₂ tensions (indicated in the text) were produced by less stringent evacuation prior to filling with H₂ and CO₂. For microaerobic growth on formate, serum agar plates were supplemented with 80mM sodium formate and N₂ gas was used in place of the hydrogen.

After growth at 37°C for 24 or 72 hours, the cells were harvested by scraping from the agar surface. The cells were washed (unless stated otherwise) in 10mM sodium phosphate pH7.

Pseudomonas stutzeri (Strain 224, ATCC 17591) was grown aerobically and under denitrifying conditions as described in Goodhew et al. (1986).

Pseudomonas aeruginosa (NCTC 10332) was grown under denitrifying conditions by the method of Wood (1978).

Preparation of cell extracts and membranes from C.mucosalis.

The procedures were carried out at 4°C. Cells were homogenised in 3 to 5 volumes of buffer (10mM Na phosphate pH7) and disrupted either by freezing and thawing twice or by passage through a French pressure cell at 82.7×10^6 Pascals (12 000 p.s.i.). Soluble and membrane fractions were routinely prepared by centrifugation at 23 000 x g for 30 minutes following freeze thawing, or 100 000 x g for 60 minutes after French press treatment. Membranes were resuspended by homogenisation in 3 to 5 volumes of buffer.

Measurements of oxygen utilisation and the detection of hydrogen peroxide formed during the oxidation of substrates.

Oxygen utilisation was measured in an oxygen electrode (Rank Brothers, Cambridge, U.K.) at 25°C in 10mM sodium phosphate (pH 7)/1mM EDTA, in a final volume of 2ml. The system was calibrated to 100% with air saturated buffer (255uM O₂) and to 0% with a few crystals of dithionite. Substrates used were

1) 1mM ascorbate with 0.3mM

N,N,N'-tetramethyl-p-phenyldiamine dihydrochloride (TMPD) (Sigma, U.K.).

2) 1mM ascorbate with 10uM cytochrome c.

Horse heart cytochrome c, type VI was from Sigma, cytochrome c-551 was prepared (K.R. Brown, Biochemistry Unit RDSVS) from Ps.stutzeri or from Ps.aeruginosa, cytochrome c-555 was prepared (G.W. Pettigrew, Biochemistry Unit RDSVS) from Chlorobium limicola f. thiosulphatophilum and cytochrome c-553 was prepared from C.mucosalis by the method of Goodhew et al. (1988).

3) 10mM sodium formate or pulses of 250nmol formate.

Hydrogen peroxide formed during these experiments was detected by the O₂ released by dismutation following the addition of 1400 Sigma units of catalase.

HOQNO (2-heptyl-4-hydroxyquinoline N-oxide, Sigma), when present, was preincubated with the cell suspension or

membranes at a concentration of 50uM.

Extraction with acid-acetone and ethanol-acetone.

Samples for electrophoresis.

To 1 volume of sample was added 9 volumes of 0.01M HCl in acetone and after 30 minutes the precipitate was sedimented in a Beckman microfuge (1 minute). The pellet was dissolved in 2% SDS/62.5mM TRIS (Tris[hydroxymethyl]aminomethane) -HCl/2mM EDTA/10% glycerol (pH 7) ready for electrophoresis.

Ethanol-acetone extraction was carried out similarly using ethanol:acetone (1:1).

Polyacrylamide gel electrophoresis (PAGE)

1) PAGE under denaturing conditions.

Electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was carried out using the buffer system of Laemmli (1970) with the addition of 2mM ethylenediaminetetraacetic acid (EDTA). Gels were cast (% acrylamide and % bisacrylamide as detailed in figure legends) as slabs (130 x 150 x 1mm) with a 4% acrylamide and 0.1% bisacrylamide stacking gel. The separating (running) gel and the electrode buffer were pH 8.8, the stacking gel was pH 6.8. Samples were pre-treated for 2 minutes with 2% SDS/62.5mM TRIS-HCl/2mM EDTA/10% glycerol (pH7), at room temperature (unless stated otherwise). Electrophoresis was performed at 50V for approximately 16 hours or at 150V for 4-5 hours.

Gels were stained for haem peroxidase activity by the method of Thomas et al. (1976) with the following modifications. Gels were immersed for 30 minutes in 1.25mM TMBZ (3,3',5,5'-tetramethylbenzidine) (Sigma) in methanol/0.25M sodium acetate (pH 5) (30:70) with constant shaking after which H₂O₂ was added to 26mM. (1.88mM TMBZ and 52mM H₂O₂ were also employed for selected experiments). After shaking for a further 15 to 30 minutes, the staining solution was replaced at least twice with fresh propanol/0.25M sodium acetate (pH 5) (30:70).

Photography and scanning of gels was carried out within 2 hours of staining. Photography of gels was through a yellow filter (Cokin A.001). The centre of each channel was scanned using a Shimadzu CS-930 gel scanner at 650 or 690nm in transmission mode with a beam of dimensions 0.05 x 2mm in 0.2mm steps.

2) Native PAGE.

The gels and buffer systems were as 1) above, but with the omission of SDS. The gel (10% acrylamide/0.3% bisacrylamide) and electrode buffer were pH 8, the 'stacking' gel (4% acrylamide/0.1% bisacrylamide) was pH 8.8. The polarity of the apparatus was reversed, compared to that used for SDS-PAGE, so that basic proteins (positively charged at pH 8) ran through the gel towards the cathode. Electrophoresis was performed at 100V for 5 hours. The gels were stained for protein with 2.0g/l Coomassie brilliant blue R in methanol/acetic acid/water (4.5:1:4.5) and destained in methanol/acetic acid/water (3:1:6) and photographed as for haem stained gels.

Protein Assays

Protein was determined as follows by a method based on Ohnishi and Barr (1978), itself a modification of the Lowry et al. (1951) biuret method. Biuret reagent (2mM CuSO_4 /3.5mM potassium sodium tartrate/0.5M NaOH/0.9M sodium carbonate) (1ml) was added to 1ml sample (containing 0 to 200ug protein) and incubated for 10 minutes at room temperature. Folin and Ciocalteu's phenol reagent (diluted 1:16 with H_2O) (4ml) was then added. Tubes were incubated for 5 minutes at 55°C, cooled on ice and, after 5 minutes, the A_{650} was determined against a reagent blank. Standard curves were prepared using 0 to 200ug bovine serum albumin (BSA, Sigma).

Cytochrome spectra

Absolute spectra were recorded on a Unicam SP1800 or a Varian Cary 219 spectrophotometer. Air oxidised, ferricyanide oxidised, ascorbate/PMS reduced and dithionite reduced spectra

were made versus 0.1M pH 7 sodium phosphate buffer. Ferricyanide oxidised spectra were produced by the addition of one crystal of potassium ferricyanide. 1 mM ascorbate (pH7)/25uM PMS (phenazine methosulphate) were used to produce ascorbate/PMS spectra. Dithionite reduced spectra were produced by the addition of a few crystals of sodium dithionite.

Difference spectra were obtained using the Cary spectrophotometer. The baseline (oxidised versus oxidised or reduced versus reduced) was recorded then dithionite reduced minus air oxidised and dithionite reduced + CO minus dithionite reduced spectra were determined. CO binding spectra were obtained by bubbling the dithionite reduced suspensions with CO gas for 15 seconds.

Tissue culture

1) Growth, maintenance and re-seeding of cells.

A tissue culture line of pig kidney cells was grown as a monolayer on Eagle's medium (Dulbeccos modification) with 10% calf serum but without antibiotics, in tissue culture bottles at 37°C. Cells were refed with maintenance medium (as the growth medium, but containing 2.5-5% calf serum) after 2 or 3 days. Seeding into new bottles was performed 1 day after refeeding. For this the tissue cells were washed and then detached from the bottles with saline trypsin versene (STV) which is phosphate buffered saline containing 0.01% trypsin and 0.01% EDTA. After centrifugation the cells were suspended in growth medium and the cell density estimated by counting in a Neubauer counting chamber (hemocytometer). Cells were seeded at approximately 1×10^5 cells per ml (10ml total in each culture bottle).

Cells for staining were grown on the surface of halved coverslips inside test tubes containing 1ml growth medium. Seeding was at a lower concentration (1×10^4 cells per ml) so that a monolayer did not form and individual cells were thus visible when viewed under the microscope.

2) Infection of tissues with C.mucosalis.

C.mucosalis was subcultured onto a blood agar slope in a McCartney bottle plugged with sterile cotton wool then grown microaerobically (as described above) with H_2 as e^- donor for 24 hours. Growth medium (10ml), at $37^\circ C$, was used to wash off the surface growth from the slope. This suspension of C.mucosalis was then used to replace the growth medium from a tissue culture monolayer, 24 hours after seeding. The incubation was continued at $37^\circ C$. No special atmospheric conditions were employed following inoculation with bacteria, the tissue culture bottles were capped firmly as before.

3) Staining with tetrazolium salts.

Coverslips with cultured cells (infected and uninfected) and C.mucosalis suspensions were incubated at $37^\circ C$ in 75mM NaCl containing 0.7mg of the tetrazolium salt per ml. Sodium succinate (40mM) or sodium formate (40mM) were present as substrates. The substrate H_2 was provided by saturating the NaCl solution with H_2 gas prior to adding the cells, then the atmosphere above the liquid was flushed with the gas before capping the tubes. The incubation was then completed (as with the other substrates) in a water bath at $37^\circ C$.

Cells were fixed by immersion in methanol (10 minutes) air dried and mounted on microscope slides with DePeX.

4) Extraction of reduced nitro-blue tetrazolium (formazans) from tissue culture cells and C.mucosalis suspensions.

2.5 volumes of 0.1M HCl in acetone was used to 1 volume of sample. The formazan concentration was determined from A_{550} readings of the supernatant fraction after centrifugation.

Preparation of mitochondria from rat liver.

Rat liver (separated from connective tissue with a scalpel) was hand homogenised in 4 volumes of ice-cold buffered sucrose solution [0.25M sucrose/10mM MOPS (3-[N-morpholino] propanesulfonic acid)/1mM EGTA

(ethyleneglycolbis-[aminoethylether] tetra-acetic acid)] then diluted to 10 volumes with the same buffer and centrifuged at 2000 x g for 15 seconds. The pellet was discarded and the supernatant centrifuged for 3 minutes at 18 000 x g. The pellet was resuspended to 3 volumes with buffer and the two centrifugation steps were repeated. The mitochondrial pellet was finally suspended to 1.5 volumes with SKH buffer [125mM sucrose/60mM KCl/3mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) pH 7.2].

Molecular exclusion chromatography.

G75 and G150 superfine Sephadex columns (80 x 2cm) were packed and run according to the manufacturers recommendations (Gel filtration theory and practice, Pharmacia Fine Chemicals). The composition of the elution buffers are described in the figure legends.

Enzyme Assays

[One International Unit (U) of enzyme activity is that which will catalyse the transformation of 1u mole of substrate per minute.]

1) Isocitrate Dehydrogenase (ICDH).

Buffer/substrate solution (10mM 3-[N-Morpholino] propanesulfonic acid [MOPS]/4.6mM D.L. Isocitrate/52mM NaCl, pH 6.5) (2.85ml) was incubated with the test sample (50ul) at 25°C for 5 minutes. The reaction was started by the addition of 0.1ml NADP/MnSO₄ to final concentrations of 0.33mM and 4mM respectively. The formation of NADPH was monitored at 340nm.

2) Malate Dehydrogenase (MDH).

Sodium phosphate (0.1M, pH 7.5)/0.5mM Oxaloacetic acid/0.2mM NADH (3ml final volume) was incubated at 25°C for 5 minutes. The addition of the test sample (50ul) started the reaction and the utilization of NADH was followed by the decrease in A₃₄₀.

3) Alkaline Phosphatase (AP).

The '30 minute' method of Boehringer Mannheim was used. To 1ml of substrate/buffer (5.5mM p-nitrophenyl phosphate/50mM glycine/0.5mM MgCl₂ pH10.5) was added 0.1ml sample. After exactly 30 minutes incubation at 37°C, 10ml of 0.02M NaOH was added and the optical density read (405nm) against a blank. This assay requires a blank to be prepared for each sample. The blank was prepared as for the test solution, except that the enzyme sample was added after the addition of NaOH.

$$\text{mU/ml} = A_{405} \times 200$$

4) Catalase.

The substrate solution (0.1ml H₂O₂ [8.8M] in 50ml 0.1M phosphate buffer, pH7) was adjusted, if necessary, by dilution to give A₂₄₀ of 0.55. The sample was added to 3ml of the substrate solution and the time taken (in minutes) for the A₂₄₀ to fall from 0.45 to 0.40 (equivalent to 3.45 umole H₂O₂) was measured.

$$\text{Sigma Units} = \frac{3.45}{\text{mins}}$$

5) Formate Dehydrogenase (FDH) and 6) Fumarate Reductase (FR).

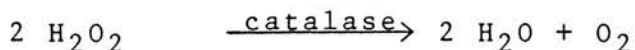
FDH and FR enzyme activities were measured in sequence by the reduction and oxidation of benzylviologen respectively. This was possible as FR activity was much higher than FDH activity and thus the oxidation of benzylviologen by FR occurred in the presence of FDH. The cuvette (3ml) was stirred and contained 250uM benzylviologen/10mM Na formate/10mM Na phosphate pH7. The contents were sparged with argon for 15 minutes prior to the assay to achieve anaerobic conditions which were then maintained with argon throughout. The sample (10ul) was added and FDH activity monitored by following the increase in A₅₄₆ as benzylviologen was reduced. FR activity was then determined from decreasing A₅₄₆ following the addition of 5mM Na fumarate. In the absence of FDH activity, benzylviologen was reduced in situ by titration with 30mM

sodium dithionite (prepared anaerobically in 100mM sodium phosphate, pH7). Na fumarate was then added and FR activity determined as above.

Chapter 3

Cytochrome-c Peroxidase

Substrate oxidation by oxygen can result in the production of superoxide radicals and H_2O_2 . These are potentially damaging species, and many bacteria contain superoxide dismutase (SOD), to convert superoxide to H_2O_2 , and catalase to detoxify H_2O_2 . Catalase catalyses the dismutation of H_2O_2 to H_2O and O_2 :-



C.mucosalis and some other Campylobacter species are catalase negative and therefore they cannot remove H_2O_2 by dismutation. C.mucosalis like the related C.sputorum ss bubulus (Niekus et al. 1980b) contains a cytochrome-c peroxidase which reduces H_2O_2 to water via cytochrome-c mediated e^- transfer from the electron transport system thus:-



The production of H_2O_2 by C.sputorum ss bubulus is thought to be raised by certain growth media and by high oxygen concentrations (Niekus et al. 1978), but cytochrome-c peroxidase activity was not induced in response to these high levels of H_2O_2 (Niekus et al. 1980b).

The cytochrome-c peroxidase has not been characterised or isolated from any Campylobacter species. In comparison the Pseudomonas aeruginosa cytochrome-c peroxidase has been isolated and fully characterised by Ellfolk and co-workers. (Ellfolk et al. 1983, 1984, Ellfolk and Soininen 1970, 1971, Soininen and Ellfolk 1972, 1973, 1975, Soininen et al. 1970, 1973).

The aims of the cytochrome-c peroxidase experiments with C.mucosalis were :-

1) The preparation of cell extracts from which the location of cytochrome-c peroxidase within the organism could be determined.

2) To develop the assay system to enable accurate measurements of cytochrome-c peroxidase activity in soluble and membrane fractions.

3) Use of the optimum assay system or systems, to measure induction of cytochrome-c peroxidase activity in response to certain growth conditions.

4) To isolate the cytochrome-c peroxidase and determine its molecular weight and nature of the prosthetic group.

5) Determination of the properties of cytochrome-c peroxidase in relation to its interaction with the e^- donor.

1) Preparation of Cell Extracts for Cytochrome-c Peroxidase Assays

To obtain precise information on the location, and the possible functions of cytochrome-c peroxidase it is desirable to produce spheroplasts of the organism. Peroxidase activity in the soluble fraction, from a spheroplast preparation, prior to lysis of the cytoplasmic membrane indicates a periplasmic location, activity after lysis implies that the enzyme is cytoplasmic. The topography of a membrane-bound enzyme as periplasmic or cytoplasmic facing, can be determined by the absence or presence respectively, of enhanced enzyme activity following lysis of the cytoplasmic membrane.

However, despite many attempts, formation of spheroplasts from C.mucosalis was not possible, because premature lysis of the cytoplasmic membrane occurred. The experiments that were carried out with the aim of forming spheroplasts are

described in chapter 6.

In the absence of spheroplast fractions, precise enzyme locations cannot be determined. Total enzyme activities can often be assessed in bacteria, by lysing the outer membrane, cell wall and cytoplasmic membrane, so facilitating access of the substrate to the enzyme regardless of its location. Separation of a total cell lysate, into membrane and supernatant fractions by centrifugation, prior to enzyme assays, will locate the enzyme as either membrane associated or as a soluble enzyme. Cytoplasmic and periplasmic locations cannot be distinguished from each other by this method.

The fragility of the outer and cytoplasmic membranes of C.mucosalis, which prevents the formation of spheroplasts, is advantageous for the preparation of total cell lysates. Many species of bacteria remain intact when frozen, and require French pressure cell treatment, sonication or lysozyme plus an osmotic shock, to lyse the membranes. With C.mucosalis the alternative method of lysis by freeze-thaw treatment is possible.

Freeze-thaw treatment of a C.mucosalis cell suspension involves freezing at -40°C and thawing twice, which from electron microscopy studies (Figure 2) has been shown to produce large 'cell sized' membranes which can easily be separated from the soluble fraction by a low speed centrifugation step of $11\,400 \times g$ for 30 minutes. The soluble extract from a freeze-thaw break was therefore membrane-free, this finding was confirmed by absence of activity from membrane marker enzymes formate dehydrogenase (FDH) and fumarate reductase (FR). The only drawback of the freeze-thaw method is that any unbroken cells will sediment with the membrane fraction, which will thus contain some trapped soluble material. In general, contamination of membranes with soluble material was very low in freeze-thaw breaks, as determined by absence of soluble cytochrome c-553 and the cytoplasmic enzymes malate dehydrogenase (MDH) and isocitrate dehydrogenase (ICDH) from the membrane fractions.

Total cell lysis was assured following passage through a

French pressure cell at 12 000 p.s.i. (82.7×10^6 Pascals) and this method was also used to prepare extracts of C.mucosalis. This method however produces many small membrane vesicles (Figure 3), which do not sediment completely even after high speed centrifugation at 100 000 x g for 1 hour. Therefore the soluble extract from a French press break always contains some membrane contamination. Centrifugation for a longer period would remove the membrane vesicles, but there is then a risk of sedimenting large, soluble proteins as well (Penefsky and Tzagoloff 1971).

2) Development of Optimum Assay System for Cytochrome-c Peroxidase

The assay, in outline, for cytochrome-c peroxidase activity consists of following spectroscopically the exponential progress curve as reduced cytochrome-c is oxidised in the presence of the enzyme and H_2O_2 .

The cytochrome c-553 from C.mucosalis is the preferred e^- donor to the C.mucosalis cytochrome-c peroxidase. The determination of specificity for e^- donor is described in section 5 of this chapter. The availability of c-553 was limited, therefore routine cytochrome-c peroxidase assays were carried out using horse heart cytochrome-c (Type VI, Sigma) as e^- donor.

Various problems were encountered in association with the cytochrome-c peroxidase assay. These included :-

a) Artifactual oxidation and reduction of horse heart cytochrome-c, attributable to the method used to prepare ferrocytochrome-c.

b) Artifactual oxidation and reduction of cytochrome-c due to components present in C.mucosalis extracts.

c) Instability of the C.mucosalis cytochrome-c peroxidase.

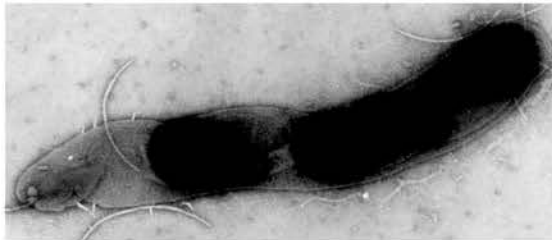


Figure 2. Electron micrograph of freeze-thaw treated C.mucosalis. Magnification x 25 000, scale line = 0.5um. The cells were harvested and frozen at -40°C for 1 hour and thawed. The preparation of E.M. grids and the staining of the samples was performed by Richard Bingham (Veterinary Pathology RDSVS) as follows. The sample was placed on the grid, blotted, washed x 2 with distilled water, blotted and stained with methylamine tungstate. Electron microscopy was performed in a Philips 400 transmission electron microscope.

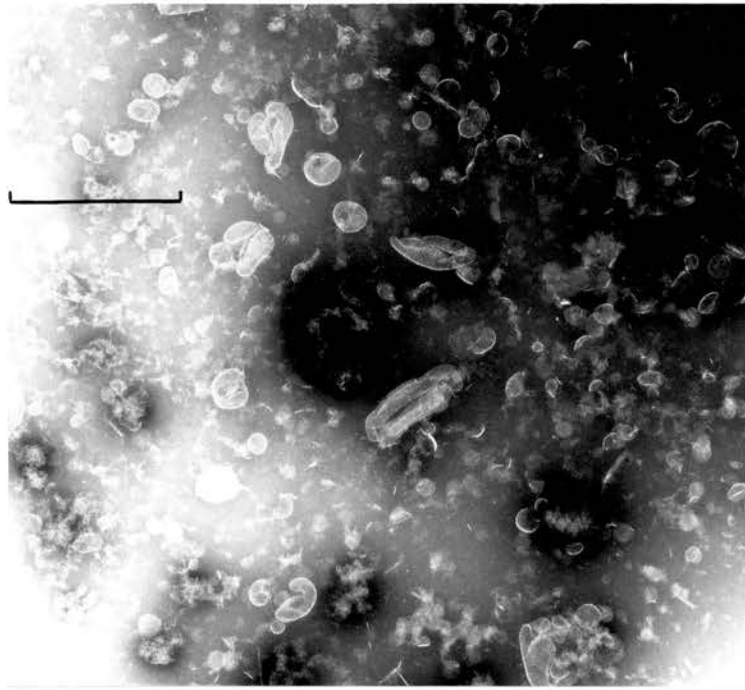


Figure 3. Electron micrograph of *C.mucosalis* after French-press treatment. Magnification x 43 000, scale line = 0.5 μ m. A cell suspension was treated by passage through a French pressure cell at 12 000 p.s.i. (82.7×10^6 Pascals). Preparation for microscopy was as described in figure 2, except that Potassium phospho-tungstate was the stain used.

a) Artifactual oxidation and Reduction of Cytochrome-c attributed to Methods used to prepare the substrate, ferrocytochrome-c.

Reduced horse heart cytochrome-c was prepared either by titration in situ with stoichiometric amounts of dithionite into a cuvette kept anaerobic by argon, or reduction was with excess ascorbate followed by desalting on Sephadex G25.

Peroxidase assays using in situ dithionite reduced cytochrome-c were associated with some oxidation of the cytochrome following sample addition, but prior to the addition of H_2O_2 (Figure 4). Dithionite reacts with O_2 in solution to produce H_2O_2 (Dixon 1971 a). The use of dithionite in air-saturated solutions to reduce cytochrome-c, results in the production of superoxide radicals, which rapidly react to form H_2O_2 in the presence or absence of SOD (Lambeth and Palmer 1973).

For peroxidase assays, argon sparged buffer was used to prepare the stock dithionite solution, which was then titrated into an argon flushed cuvette to reduce the cytochrome-c. The system should therefore be anaerobic, however the total elimination of O_2 from such systems, to prevent H_2O_2 production, involves extreme measures (Dixon 1971 a). It seems that traces of oxygen were present in our system, resulting in H_2O_2 production and hence the 'premature' peroxidative oxidation of cytochrome-c on addition of a peroxidase sample. That hydrogen peroxide production occurs in these conditions, from traces of O_2 reacting with dithionite, has been determined by using scrupulously anaerobic conditions which minimised the effect. This conclusion was confirmed by elimination of the effect, when catalase was present to convert any H_2O_2 formed into O_2 and H_2O . The peroxidase assay itself cannot, of course, be carried out in the presence of catalase.

As dithionite was associated with these problems, the reduction of cytochrome-c with ascorbate was the method of

choice for the peroxidase assays. Ascorbate had then to be removed from the cytochrome-c by desalting, before the latter could be used as e^- donor to the peroxidase.

The presence of ascorbate during the assay inhibited the peroxidative oxidation of cytochrome-c, even when present at 30uM, the concentration of ascorbate used by Berry and Trumpower (1985) for cytochrome-c oxidase assays. These authors claimed that the rate of cytochrome-c re-reduction was negligible at this concentration of ascorbate. This was not in agreement with my observations, which suggested that re-reduction was affecting the measured peroxidase activity.

The inhibition seen with excess ascorbate may however be due to the destruction of the H_2O_2 used in the peroxidase assay (Dixon 1971b). H_2O_2 absorbs at 240nm, so its destruction by ascorbate should be accompanied by falling optical density. Unfortunately, ascorbate itself also absorbs strongly at 240nm, so masking any changes due to disappearance of H_2O_2 .

After desalting, to remove ascorbate, reduced horse heart cytochrome-c auto-oxidises in air only very slowly, which does not interfere with the peroxidase assay. Peroxidase activity measured under anaerobic conditions using in situ dithionite reduction, or ascorbate reduction and desalting of horse heart cytochrome-c, gave virtually identical results (Figure 5). The latter method became the routine method used.

The peroxidative oxidation of cytochrome-c follows an exponential progress curve of decreasing absorbance, measured at the α -peak (550nm for horse heart cytochrome-c) Pseudo-first order rate constants were derived from plots of $\log A_t - A_\infty$, against time, where A_t is the absorbance of the α -peak at time t, and A_∞ is the absorbance after addition of ferricyanide to fully oxidise the cytochrome-c. Velocities were derived from the initial reaction rate or (more reliably) were calculated from $v = k \times [C_r]$, where k is the pseudo-first order rate constant, and $[C_r]$ is the concentration of ferrocytochrome-c.

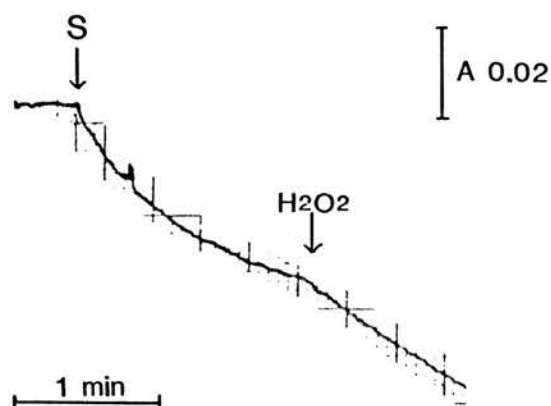


Figure 4. Premature oxidation of ferrocytochrome-c during cytochrome-c peroxidase assays, following sample addition. The anaerobic, stirred cuvette contained 6uM horse cytochrome-c/1mM EDTA/20mM Na phosphate (pH7) which was flushed with argon for 10 minutes prior to the reduction of the cytochrome in situ by titration with 10mM sodium dithionite (prepared anaerobically in 20mM sodium phosphate, pH7). Oxidation of the cytochrome-c was monitored at 550nm and occurred following the addition (S) of a freeze-thaw supernatant (25ul) from C.mucosalis. H₂O₂ was added to a final concentration of 17uM.

Cytochrome-c Peroxidase

Log $A_t - A_\infty$ (inf)

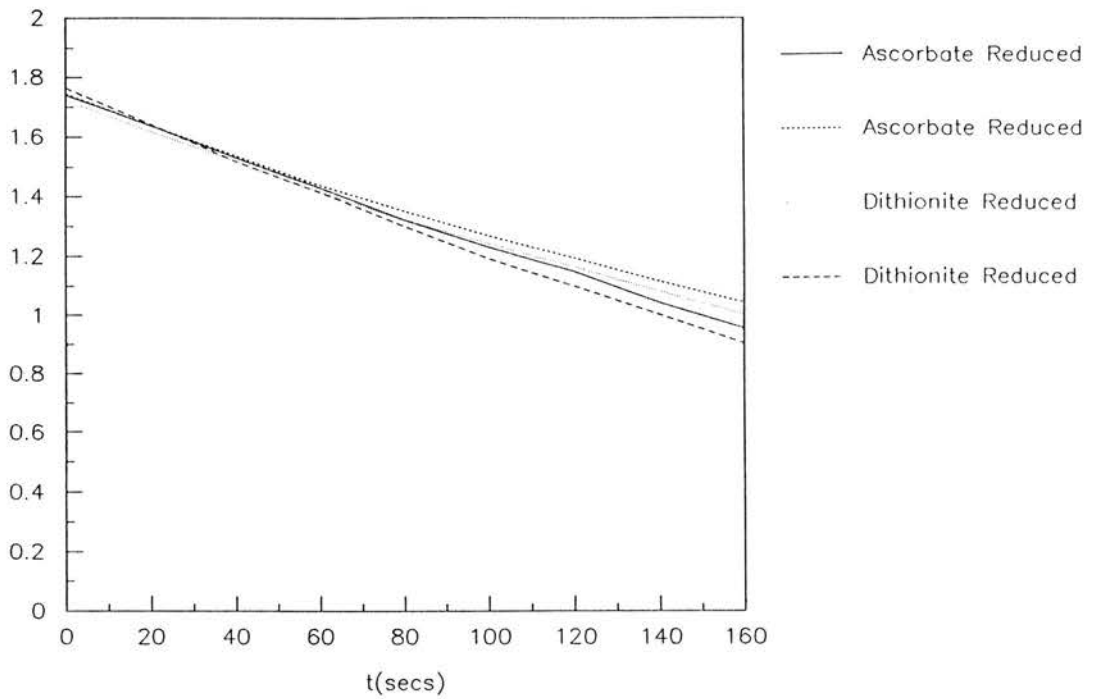


Figure 5. Comparison of the cytochrome-c peroxidase assay using alternative methods for the preparation of ferrocytochrome-c. The cuvette was kept anaerobic and contained 6uM horse cytochrome-c/1mM EDTA/10mM Na phosphate, pH7. The cytochrome was reduced with ascorbate or dithionite. 1mM ascorbate was added to reduce the cytochrome and after 5 minutes on ice the cytochrome was desalted on G25 Sephadex (1 x 10cm column) into 10mM Na phosphate/1mM EDTA, pH7, to remove the reductant. Dithionite reduction was performed in situ by titration with 10mM sodium dithionite as described in figure 4.

H_2O_2 was added to a final concentration of 17uM. The reaction was started by the addition of 10ul of a freeze-thaw supernatant (derived from a broken cell suspension containing 20mg protein per ml). The (semi-log) plot was derived from $\text{Log } A_t - A_\infty$ against time, where A_t = absorbance of the α -peak (550nm) at time t, and A_∞ = absorbance following the addition of K ferricyanide.

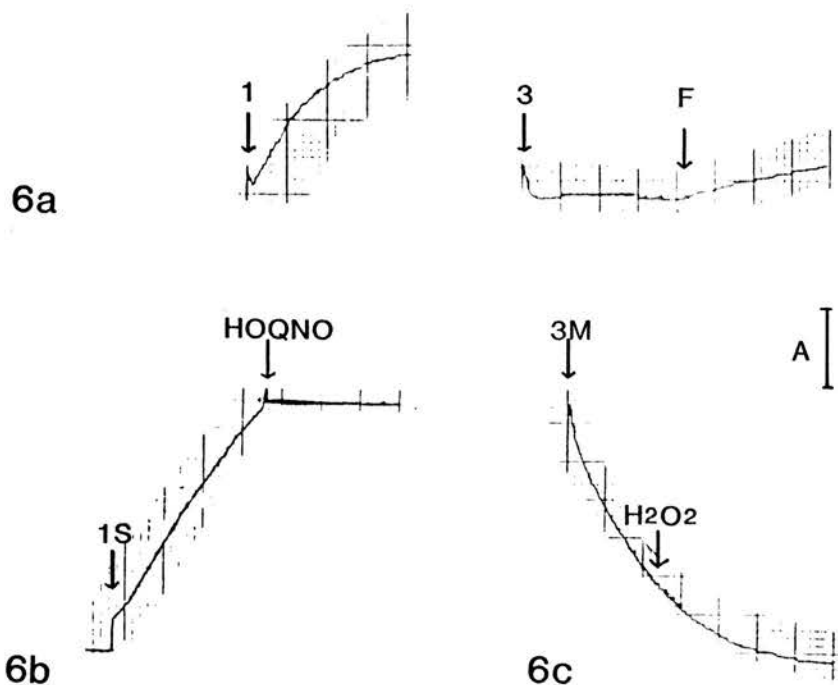


Figure 6. Interference by oxidation and reduction of cytochrome-c during cytochrome-c peroxidase assays. The anaerobic cuvette contained 10mM Na phosphate (pH7), 1mM EDTA and 6a,6b 7.0uM oxidised horse cytochrome-c, 6c 5uM ascorbate reduced and desalted horse cytochrome-c. All *C.mucosalis* extracts were derived from broken cell suspensions containing 20mg protein per ml. 6a Cytochrome-c was reduced following the addition of 10ul whole 1 day cell suspension alone (1), or 10ul whole 3 day suspension (3) after the addition of 80uM formate (F). 6b Cytochrome-c reduction by 10ul 1 day French-press supernatant (1S) was halted by the addition of HOQNO to a final concentration of 33uM. In 6c, cytochrome-c oxidation proceeded in the presence of 10ul of a washed 3 day membrane suspension (3M). H₂O₂ was added to a final concentration of 17uM. Absorbance scale (A) = 0.05 (figure 6a) and 0.02 (figure 6b and 6c)

b) Artifactual oxidation and Reduction of Cytochrome-c due to components in C.mucosalis extracts.

Under aerobic conditions, with French press supernatants, the oxidation of ascorbate reduced and desalted horse heart cytochrome-c occurred, prior to the addition of H_2O_2 . This was due to the presence of small membrane vesicles, which possess cytochrome-c oxidase activity. The effect was minimised with French press supernatants, under anaerobic conditions. Cytochrome-c oxidase activity was not seen with Freeze-thaw supernatants, even in aerobic assays, as these preparations were membrane-free, as described in section 1.

Anaerobic conditions did not, however, eliminate the substantial levels of cytochrome-c oxidation which occurred with whole cell lysates and membrane suspensions, prior to H_2O_2 addition. Prolonged flushing of the cuvette contents with argon before the addition of a membrane suspension, failed to prevent unacceptably high levels of cytochrome-c oxidase activity. This was in spite of using copper tubing to convey argon to the cuvette, as it has been shown (Dixon 1971 a) that diffusion of considerable quantities of O_2 into argon occurs when it is passed through any type of polythene tubing. The cytochrome-c oxidase activity observed was therefore likely to be due to low levels of oxygen remaining or leaking into the cuvette, combined with the very high affinity that the C.mucosalis oxidase has for O_2 . (Chapter 4.)

The presence of the respiratory inhibitor KCN eliminated cytochrome-c oxidase activity. Cyanide inhibits by complexing with metallo-enzymes and will therefore also bind slowly to cytochrome-c and probably the peroxidase itself. The cytochrome-c peroxidase of C.sputorum ss bubulus (Niekus et al. 1980a) and of Ps.aeruginosa (Soininen and Ellfolk 1973) are sensitive to cyanide, and so KCN cannot be used during cytochrome-c peroxidase assays.

Reduction of cytochrome-c by cell suspensions and membranes also occurred, but was extremely variable. Some

samples rapidly reduced oxidised cytochrome-c, others reduced cytochrome-c only slowly or not at all. Extracts from C.mucosalis grown for 1 day generally exhibited higher levels of cytochrome-c reductase activity, than did cells grown for 3 days, the latter sometimes only reducing cytochrome-c following the addition of formate (Figure 6a). Soluble extracts alone never reduced cytochrome-c, but could do so after the addition of membranes. These findings suggested that soluble endogenous reductants in C.mucosalis were supplying a membrane-bound reductase with reducing equivalents. Dialysing suspensions or using washed membranes, to remove these endogenous reductants, had little effect as significant reduction of cytochrome-c still occurred. The inclusion of HOQNO did halt the reduction of cytochrome-c by suspensions (Figure 6b), and the presence of HOQNO did not appear to significantly inhibit peroxidase assays, when tested on a soluble extract.

The high cytochrome-c oxidase activity remained, however, as an unsolved problem of cytochrome-c peroxidase assays, with samples of membrane suspensions. The rate of cytochrome-c oxidation by washed membranes under 'anaerobic' conditions, was only increased slightly by the addition of H_2O_2 (Figure 6c). This suggested that the peroxidase activity associated with membranes was very low, compared to the high levels of peroxidase activity found in the soluble fraction. Therefore peroxidase assays were subsequently confined to soluble extracts of C.mucosalis.

c) Instability of the Soluble Cytochrome-c Peroxidase.

Loss of cytochrome-c peroxidase activity frequently occurred, following the preparation of a soluble cell extract. Investigations were carried out to find the cause and attempt to eliminate the loss.

The cytochrome-c peroxidase of Ps.aeruginosa, is known to be inactivated by H_2O_2 (Ronnberg et al. 1981). In C.sputorum ss bubulus H_2O_2 production, from the oxidation of formate, is

enhanced at atmospheric levels of oxygen (Niekus 1980b). Microaerophilically grown C.mucosalis was exposed to atmospheric O₂ during harvesting, therefore it seemed possible that H₂O₂, produced from the oxidation of endogenous substrates, was damaging the peroxidase and leading to its loss of activity.

The cytochrome-c peroxidase seemed to be less stable in extracts from cells grown with formate, instead of hydrogen, as e⁻ donor. During one experiment, the ratio of peroxidase activity in extracts of formate grown compared to hydrogen grown cells, fell from 4:1 to 2:1 within one to two hours. Formate, unlike H₂, was likely to be carried over from the media with the cells during harvesting, and on exposure to atmospheric O₂ may, as with C.sputorum ss bubulus, lead to the production of high levels of damaging peroxide. Experiments were conducted to determine if the presence of H₂O₂ was responsible for the loss of cytochrome-c peroxidase activity in C.mucosalis.

The addition of exogenous H₂O₂ to a soluble extract did indeed speed the loss of peroxidase activity (Figure 7a). The soluble peroxidase was protected by the presence of membranes (Figure 7b), which may allow electron transport to the peroxidase and hence removal of the H₂O₂. Following centrifugation and separation into membrane and soluble extracts, this process of detoxifying H₂O₂ is lost in the soluble fraction. French press supernatants, which contain small membrane vesicles, did appear to retain peroxidase activity rather better than the membrane-free freeze-thaw supernatants. Desalting a soluble extract to remove endogenous substrates, and any H₂O₂ produced from them, did not improve peroxidase stability, although damage may have occurred before the desalting procedure.

The soluble cytochrome-c peroxidase was found to be at higher levels and more stable in extracts from cells grown on serum plates, compared to blood plates. Blood agar plates contain erythrocyte catalase which may scavenge H₂O₂ produced by the cells during growth. This medium represses peroxidase

activity in C.mucosalis (as will be described in section 3 of this chapter). At harvesting, these cells are simultaneously exposed to high oxygen tensions, and therefore to potentially high levels of H_2O_2 production, while being separated from the protection conferred by the catalase in the medium. C.mucosalis grown on serum plates, and therefore in the absence of catalase, have higher levels of peroxidase to detoxify the H_2O_2 .

The levels of cytochrome-c peroxidase are not the only factor however, as cells from blood plates grown at a high oxygen tension (50% of atmospheric), contained higher levels of peroxidase than was present in cells grown at a lower oxygen tension (20% of atmospheric). The peroxidase was more unstable in the former extract (Figure 7a).

The stability of cytochrome-c peroxidase from cells grown on blood plates was extremely variable and remains an unresolved problem. The ultimate degree of peroxidase stability may be dependant upon the particular levels of endogenous reductants present in the cells at harvesting, and hence their availability for oxidation to form hydrogen peroxide on exposure to atmospheric oxygen.

Instability was much less of a problem with extracts from serum grown C.mucosalis, possibly due to the enhanced peroxidase activity and adaptation to high H_2O_2 levels.

3) Induction of Cytochrome-c Peroxidase activity in response to growth conditions

Catalase-negative Campylobacters, such as C.mucosalis and C.sputorum ss bubulus remove H_2O_2 by cytochrome-c peroxidase activity. Growth conditions, such as high O_2 tensions and the presence of formate, lead to enhanced H_2O_2 production in C.sputorum ss bubulus (Niekus et al. 1978), which might be expected to induce increased levels of cytochrome-c peroxidase activity. Growth media, containing peroxide-scavenging catalase may, conversely, repress peroxidase activity.

The aim of experiments with C.mucosalis, was to determine if peroxidase activity was induced in response to growth conditions where H_2O_2 levels might be expected to be high. Peroxidase activities were expressed as specific activity, to take account of any differences in cell yield.

Three aspects of growth conditions were investigated:-

- a) Effect of O_2 concentration.
- b) Effect of formate.
- c) Effect of catalase.

a) Effect of falling O_2 concentration during growth, or the effect of age of cells.

In C.sputorum ss bubulus, exposure to atmospheric levels of O_2 , from previously microaerophilic conditions, did not lead to the induction of higher levels of peroxidase activity (Niekus et al. 1980b). The exposure was rapid and extreme, and therefore it seems probable that insufficient time was available for the organism to adapt to increased H_2O_2 production, by induction of peroxidase activity. This resulted in destruction, by H_2O_2 , of the peroxidase and of the formate-oxidising enzyme (Niekus et al. 1980b).

C.mucosalis cells were grown in sealed culture jars, initially containing O_2 at around 20% to 25% of atmospheric. The oxygen concentration falls gradually during growth, due to utilisation for cell respiration. Cells harvested after 3 days growth will, therefore, have been exposed to lower oxygen tensions, than experienced by cells harvested after only 1 days growth.

Soluble cytochrome-c peroxidase levels in 1 day cells were, on average, 4.2 times higher than in 3 day cells. The comparison was made from initial rates of horse heart cytochrome-c oxidation, by 10ul of supernatants prepared from suspensions containing 20mg cellular protein per millilitre. 14 pairs of assay comparisons, from 5 sets of experimental growths on blood agar plates were analysed statistically, using Student's t test. The t value of 2.79 with n-1 (= 13)

degrees of freedom, shows that the means were significantly different, with a confidence limit of 98%, $0.02 > P > 0.01$ (Table 1 below).

Table 1

The effect of culture age on peroxidase activity.

	$\frac{\text{nmol HHC min}^{-1}}{\text{mg cellular protein}^{-1}}$	t-test
1 day	25.2 ^a	t=2.79 d.f.=13
3 day	5.9 ^a	P<0.02

^a Means from 14 pairs of assay comparisons.

Therefore as the oxygen concentration falls, or as the cultures age, cytochrome-c peroxidase activity declines.

C.mucosalis was grown on agar plates, as it cannot be grown in liquid culture in our laboratory. A constant fixed oxygen tension, which could easily be achieved in liquid culture by sparging with O₂ / N₂ gas mixtures, cannot be maintained in the sealed jars. Therefore it is difficult to separate the effects of falling O₂ concentrations, from the effects of culture age.

An attempt was made to maintain O₂ concentrations in the jars, at or near the initial level for 3 days, by regassing daily with O₂ / H₂ / CO₂. Peroxidase activity was no higher than in cells grown for 3 days under conditions of falling O₂ concentration. This might suggest that the principal factor determining peroxidase activity was in fact the age of the cells, rather than O₂ concentrations present during their growth. However, cells grown for 1 day at an initially 'high' oxygen concentration of 50% of atmospheric, did exhibit higher levels of peroxidase activity than was found in 1 day cells grown with 'low' initial O₂ concentrations of only 20% of atmospheric (Figure 7a. Therefore, relatively high O₂ concentrations can induce cytochrome-c peroxidase activity, at least during the early stages of cell growth.

Figure 7. Instability of soluble cytochrome-c peroxidase. The cuvette contained 6uM reduced horse cytochrome-c/1mM EDTA/20mM Na phosphate (pH7). The samples were all 1 day Freeze-thaw supernatants. Zero time (except in the case of Non-desalted 2) was the time at which the supernatant was separated from the membranes. Non-desalted 2 was separated from membranes at 180 minutes.

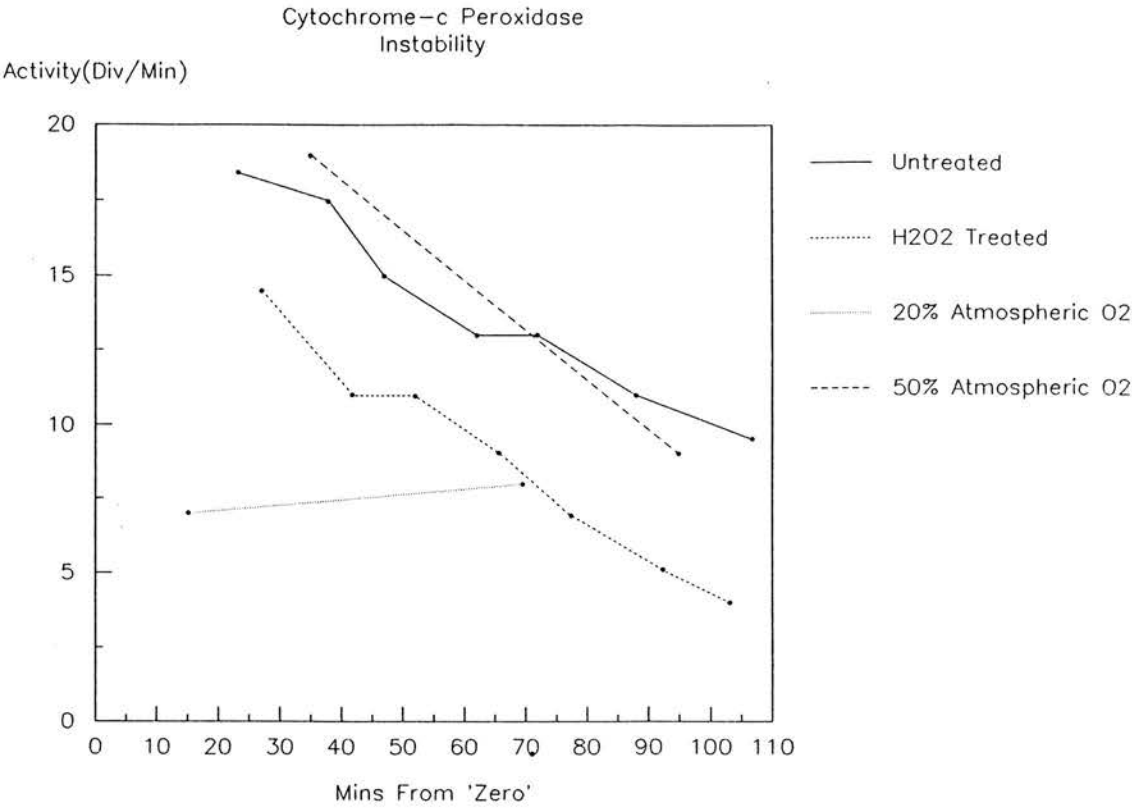
Experiment 1. The H_2O_2 treated supernatant had 1mM H_2O_2 added 5 minutes after zero. For the peroxidase assay, 10ul of the H_2O_2 -treated and untreated supernatants were used.

Experiment 2. 20% and 50% of atmospheric O_2 were the initial O_2 concentrations present in the microaerobic growth jars. 25ul samples of these two supernatants were used for the assay.

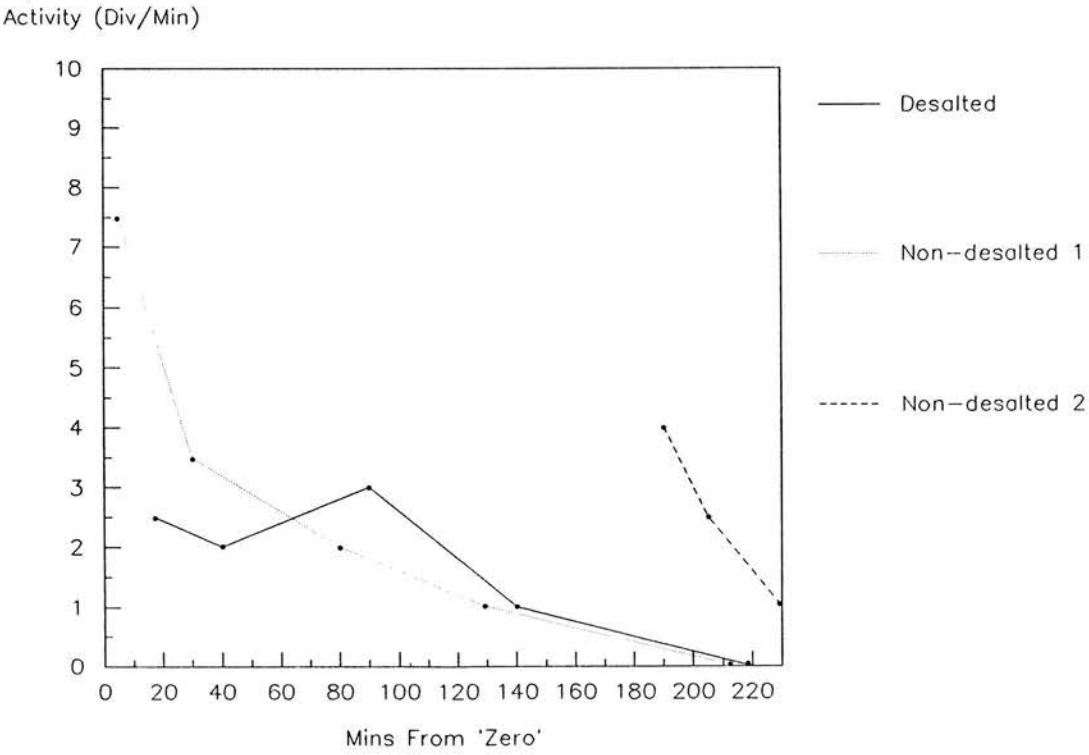
Experiment 3. The desalted supernatant was desalted into 10mM Na phosphate (pH7) on a G25 Sephadex column (1 x 10cm). The volume of the sample used for the peroxidase assay was adjusted to take account of the dilution that occurred due to the desalting procedure.

The peroxidase activities were derived from the initial reaction rate. 1 division per minute is equivalent to the oxidation of 0.3nmol horse cytochrome-c per minute.

a)



b)



b) Induction of Cytochrome-c peroxidase activity in the presence of Formate.

C.sputorum ss bubulus (Niekus and Stouthamer 1981) and C.mucosalis (chapter 6 this thesis/Goodhew et al. 1988) have been shown to produce H_2O_2 as well as H_2O from the oxidation of formate.

C.mucosalis was grown microaerobically, with H_2 or formate as e^- donor. Sodium formate (80mM) was supplied as a media supplement to serum agar plates, under a $N_2 / CO_2 / O_2$ atmosphere. For H_2 growth, formate was omitted from the medium, and gaseous H_2 replaced the nitrogen.

5 pairs of assay comparisons from 2 sets of experimental growths were assayed for cytochrome-c peroxidase activity, which was 1.5 to 4 times higher in cells grown with formate compared to H_2 as e^- donor. These differences were likely to be underestimates, due to the instability of the peroxidase from formate grown C.mucosalis, as described in section 2c. Analysis using Student's t-test, however, showed that the means were significantly different with a confidence limit of 99% (Table 2 below).

Table 2

The effect of electron donor on peroxidase activity.

<u>Electron donor</u>	<u>nmol HHC min⁻¹ mg cellular protein⁻¹</u>	<u>t-test</u>
Formate	46.2 ^a	t=5.87 d.f.=4
Hydrogen	24.7 ^a	P<0.01

^a Means from 5 pairs of assay comparisons.

d.f. = degrees of freedom

HHC = Horse heart cytochrome-c

This result is consistent with the theory of enhanced H_2O_2 production, from formate oxidation, inducing higher levels of cytochrome-c peroxidase activity.

c) Repression of Cytochrome-c peroxidase activity in the presence of Catalase.

Blood agar plates contain erythrocyte catalase, equivalent to approximately 450 Sigma Units per millilitre, serum agar plates do not. [1 Sigma unit decomposes 1.0umol H_2O_2 per minute at pH 7.0, 25°C.] C.mucosalis harvested from blood plates will therefore have grown in the presence of this peroxide scavenger. Under microaerobic levels of O_2 , the presence of erythrocyte catalase repressed cytochrome-c peroxidase activity to 25% of the level found in its absence, although the results were only just statistically significant (Table 3 below).

Table 3

The effect of catalase in the growth medium on peroxidase activity

	$\frac{\text{nmol HHC}}{\text{mg cellular protein}} \text{ min}^{-1}$	t-test
Serum agar	23.5 ^a	t=24.51
Blood agar	6.1 ^a	d.f.=1 P<0.05

^a Means from 2 pairs of assay comparisons.

Cells were harvested after 1 days growth within the same jar, giving identical gaseous conditions throughout, therefore the medium was the only variable. Removal of H_2O_2 by catalase appears to inhibit the induction of cytochrome-c peroxidase, a result which is not too surprising. However the growth of C.mucosalis in the presence of catalase was also inhibited by 50%, which is rather more interesting. H_2O_2 production, and its removal via the cytochrome-c peroxidase, may therefore be part of an energy conserving pathway. Experiments to determine the possible role of H_2O_2 in energy conservation will be discussed in chapter 6.

4) Isolation of Soluble Cytochrome-c peroxidase

To determine the nature and properties of C.mucosalis cytochrome-c peroxidase, it is necessary to purify the enzyme. Molecular weight (M_r) determinations can then be made, and the nature of the enzyme prosthetic group elucidated.

The aims of the C.mucosalis experiments were to purify the cytochrome-c peroxidase using Sephadex gel exclusion chromatography, followed by SDS polyacrylamide gel electrophoresis, to determine its M_r .

The cytochrome-c peroxidase assay using horse heart cytochrome-c as e^- donor is very time consuming, and uses considerable quantities of cytochrome-c. Therefore it was not feasible to use this method to assay all fractions from a chromatographic separation, in order to determine those possessing cytochrome-c peroxidase activity. A rapid preliminary screening method was required, to identify those fractions which potentially contained cytochrome-c peroxidase activity. These selected fractions alone would then be assayed specifically for cytochrome-c peroxidase activity.

a) Development of ABTS peroxidase assay for screening chromatography fractions

The screening assay used was an adaptation of the Boehringer-Mannheim GOD-Perid method for the determination of glucose levels. Glucose is estimated after oxidation catalysed by glucose oxidase to form gluconate and H_2O_2 , which then reacts with horse radish peroxidase to peroxidatively oxidise the chromogen ABTS (2,2'-Azino-bis 3-ethylbenzthiazoline sulfonic acid) to form a coloured compound.

This method was adapted for bacterial extracts, by using the bacterial peroxidase and exogenous H_2O_2 to oxidise the ABTS. The reaction could be carried out in vitro, using 1.2mM ABTS, 58uM H_2O_2 , 0.1M pH7 sodium phosphate, and the colour read in a spectrophotometer at 610nm. The method was tested with dilutions of yeast cytochrome-c peroxidase (YCCP) and of

C.mucosalis extracts, containing cytochrome-c peroxidase activity, which showed that lower concentrations of H_2O_2 were rate limiting. Incubating the reaction tubes for 25 to 50 minutes at room temperature (circa $20^{\circ}C$) gave a linear plot of peroxidase activity versus A_{610} , up to an absorbance of 0.7 (Figure 8a). Incubation at $30^{\circ}C$ or $37^{\circ}C$ inhibited the reaction, which was then also non-linear (Figure 8b).

This in vitro assay required around 1ml of a chromatography fraction, which was quite wasteful of material. The time taken to spectroscopically read alternate assay fractions, from an entire chromatographic profile, was around 1/2 to 3/4 of an hour, during which the colour can continue to develop, or begin to fade.

Therefore the method was adapted into a 'micro' assay which required just 50ul of sample and 100ul of ABTS / H_2O_2 / Pi reagent, with final concentrations as above. The reaction was carried out in a flat-bottomed microwell plate, and the colour produced by oxidation of ABTS detected using an automatic plate reader fitted with a 405nm filter. Detection of ABTS peroxidase activity at 405nm was possible, as the spectrum of ABTS product minus substrate, showed a peak at 420nm with a trough at 480nm (Figure 9a). The microwell ABTS method was rapid, linear with YCCP from a 405nm absorbance of 0.005 to 0.13 (Figure 9b), used small volumes of sample and reagent and facilitated the screening of all fractions from a Sephadex chromatography profile. The microwell ABTS peroxidase assay was very sensitive, easily detecting (with an O.D. of 0.02) activity in a YCCP sample, that gave an initial rate, for cytochrome-c peroxidase activity, of 3.0 nmole horse heart cytochrome-c oxidised per minute.

The only drawback of the microwell ABTS method was that, unlike the test-tube method, some reactivity occurred with haem proteins that were, in reality, without cytochrome-c peroxidase activity. This was probably due to partial denaturation of the protein, on binding to the plastic wells, thus exposing the haem, which then exhibited peroxidase activity with ABTS. This is analagous to the haem staining

method for detection of c-type cytochromes on SDS polyacrylamide gels (see chapter 5), in which the haem-c, denatured by SDS, possesses peroxidase activity which oxidises TMBZ in the presence of H_2O_2 , to form an insoluble coloured compound.

The consequence of this reactivity between ABTS and haem proteins, on microwell plates, was that some additional fractions were selected for cytochrome-c peroxidase assays. However, this ensured that all fractions with possible peroxidase activity were tested. The test-tube ABTS peroxidase method was used initially for screening G150 Sephadex fractions. The microwell ABTS method was subsequently adopted for screening Sephadex G75 fractions for peroxidase activity.

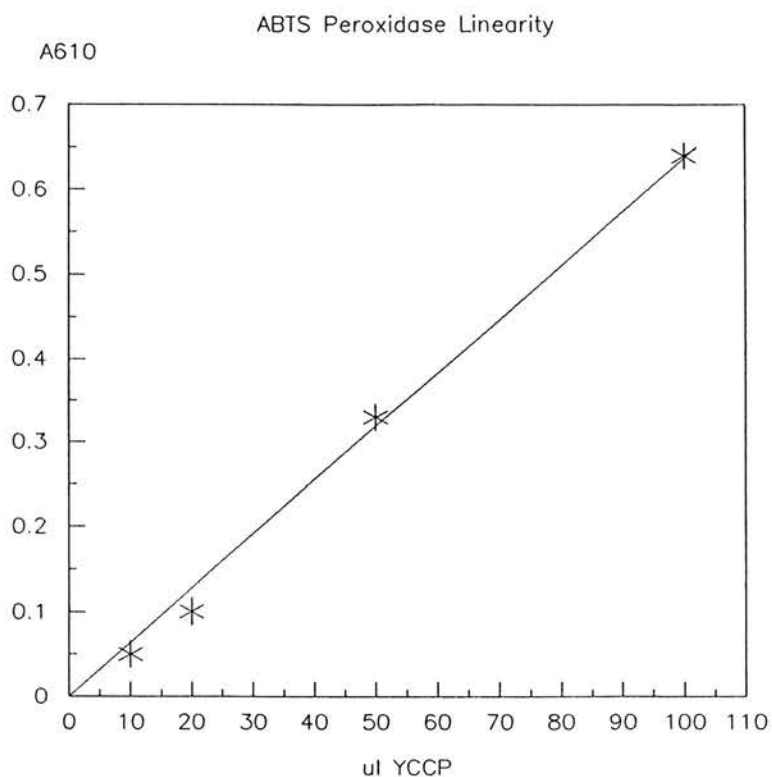


Figure 8a. The linearity of the ABTS peroxidase assay. The reaction was carried out at room temperature. The assay mixture contained 1.2mM ABTS, 58uM H_2O_2 , 0.1M Na phosphate (pH7) and 10, 20, 50 or 100ul of yeast cytochrome-c peroxidase (YCCP). The absorbance was read at 610nm after 25 minutes against a reagent blank.

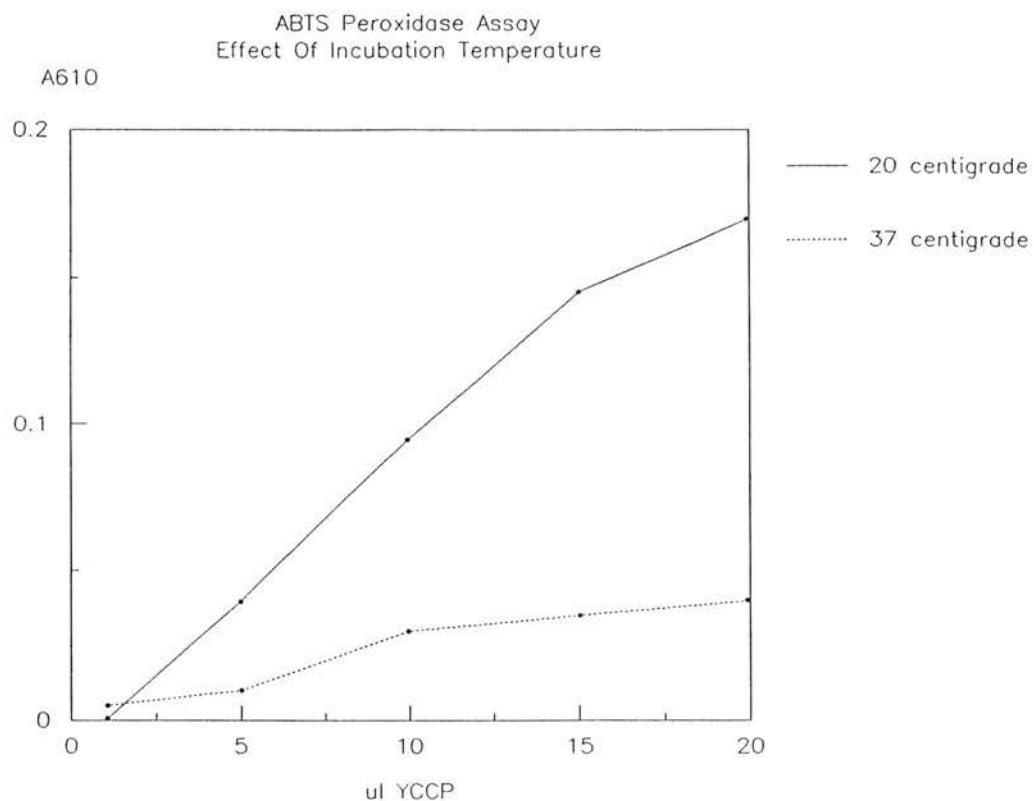


Figure 8b. The effect of incubation temperature on the ABTS peroxidase assay. The assay mixture was as described in figure 8a. Tubes containing 1, 5, 10, 15 or 20ul YCCP were then incubated at room temperature (20°C) or 37°C for 25 minutes and the absorbance read as for figure 8a.

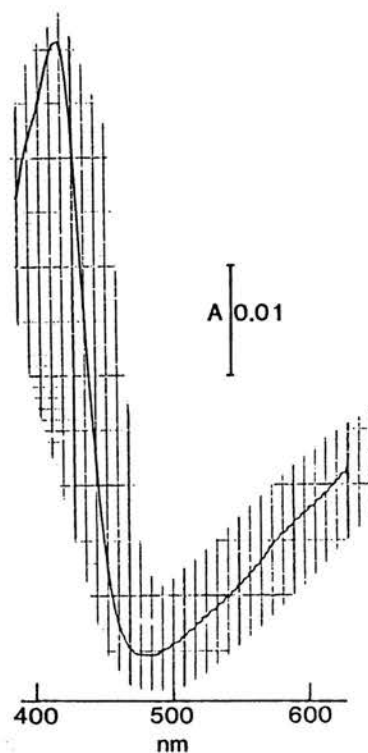


Figure 9a. Spectrum of ABTS product minus substrate. The assay was as described in figure 8a. After development of the ABTS peroxidase reaction, the spectrum of the ABTS product was recorded against a reagent blank (ABTS substrate).

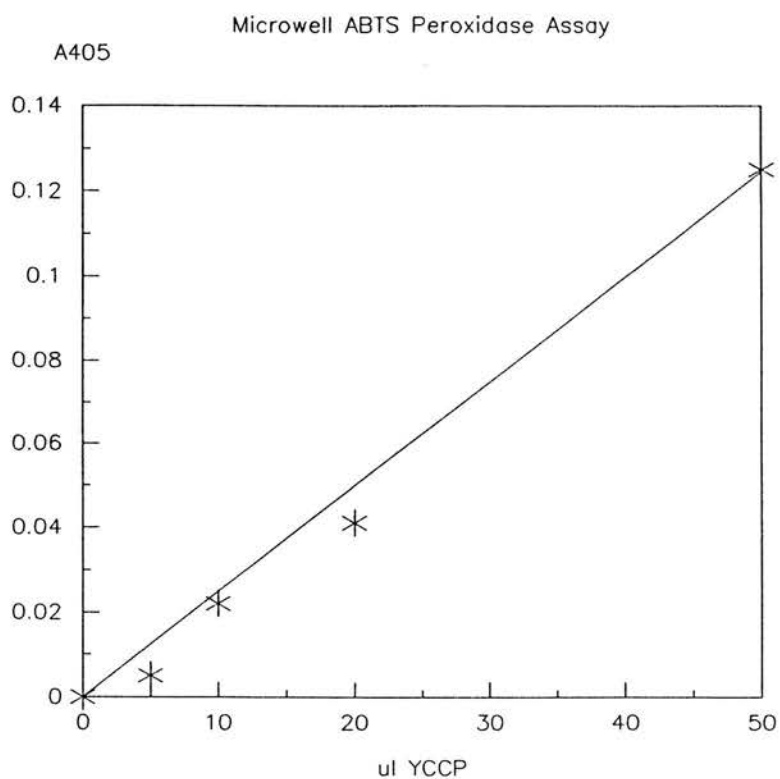


Figure 9b. Linearity of microwell ABTS peroxidase assay. Substrate concentrations were as described in figure 8a. The reaction (50ul sample, 100ul substrate) was carried out in a flat-bottomed microwell plate. The oxidised ABTS product was detected using a Titertek Multiskan microtiter plate reader fitted with a 405nm filter. The absorbance was read against a reagent blank.

b) Sephadex Gel Exclusion Chromatography to isolate the soluble cytochrome-c peroxidase from C.mucosalis

A column (2 x 80cm) of G150 superfine Sephadex, equilibrated with 5mM Tris-Cl / 100mM NaCl pH8 at 4°C, was initially used to purify the cytochrome-c peroxidase from a soluble extract of C.mucosalis. The profile exhibited two peaks with ABTS peroxidase activity, one at or near the void volume, the other at $M_r \approx 70,000$ (Figure 10). Both ABTS peroxidase peaks corresponded to haem proteins, which were monitored by 410nm absorbance of the untreated column fractions. Neither of the peaks had significant levels of horse heart cytochrome-c peroxidase activity (Figure 10), even though the ABTS absorbance readings were sufficiently high as to suggest that cytochrome-c peroxidase would be detectable.

There were several possible explanations for this, including the instability of the cytochrome-c peroxidase, a problem which has already been discussed in section 2c of this chapter. Other possible causes were proteolysis during the several days taken at 10°C to run the column, or to separation of the cytochrome-c peroxidase from a necessary co-factor.

The principal problem, however, was found to be the high ionic strength of the eluting buffer, which was almost totally inhibiting horse-heart cytochrome-c peroxidase activity. The ionic strength dependence of cytochrome-c peroxidase was determined, and will be described in section 5a of this chapter.

The presence of 5mM Tris-Cl (pH8 at 4°C) was determined to be the least inhibitory of HHC peroxidase activity, while still retaining buffering capacity. Separation of the C.mucosalis cytochrome-c peroxidase on G150 equilibrated with this low ionic strength buffer, gave a broad peak with ABTS peroxidase activity which also exhibited high levels of HHC peroxidase activity. This finding suggested that proteolysis, peroxidase instability or separation from co-factors, were not

fundamental problems of the chromatographic separation. The resolution of the column was however poor, and calibration with standard proteins was not possible, as binding of proteins to the Sephadex matrix occurs at this low ionic strength (Pharmacia booklet 'Gel Filtration Theory and Practice').

The strategy then adopted was to calibrate the column, and separate extracts on a G75 column equilibrated with a high ionic strength buffer, followed by 280nm, 410nm, and microwell ABTS peroxidase screening of fractions. Peak fractions of protein (280nm), haem proteins (410nm) and fractions with ABTS peroxidase activity, were then dialysed against a buffer of low ionic strength, prior to assays of HHC peroxidase activity.

The G75 Sephadex profile (Figure 11) revealed peaks near the void volume and at 66 000 M_r , which were haem containing and exhibited ABTS peroxidase activity. Cytochrome-c peroxidase activity was confined to the 66 000 M_r peak alone. The conclusion was that the cytochrome-c peroxidase of C.mucosalis runs on Sephadex with an apparent M_r of 66 000, and may also possess a haem centre.

The cytochrome-c peroxidase in C.mucosalis was therefore of similar M_r to the Ps.aeruginosa cytochrome-c peroxidase, which gave a M_r of 58 000 when separated on thin layer G100 Sephadex (Ellfolk and Soininen 1971). On SDS polyacrylamide gels, the Ps.aeruginosa cytochrome-c peroxidase gave a M_r of 40 000 (Soininen et al. 1973), and this enzyme was found to possess two c-type haems per molecule (Ellfolk and Soininen 1971). The higher molecular weight seen on Sephadex may reflect a rapid monomer dimer equilibrium.

Molecular weight determinations, on the C.mucosalis cytochrome-c peroxidase, were therefore made using SDS polyacrylamide gels stained for haem-c.



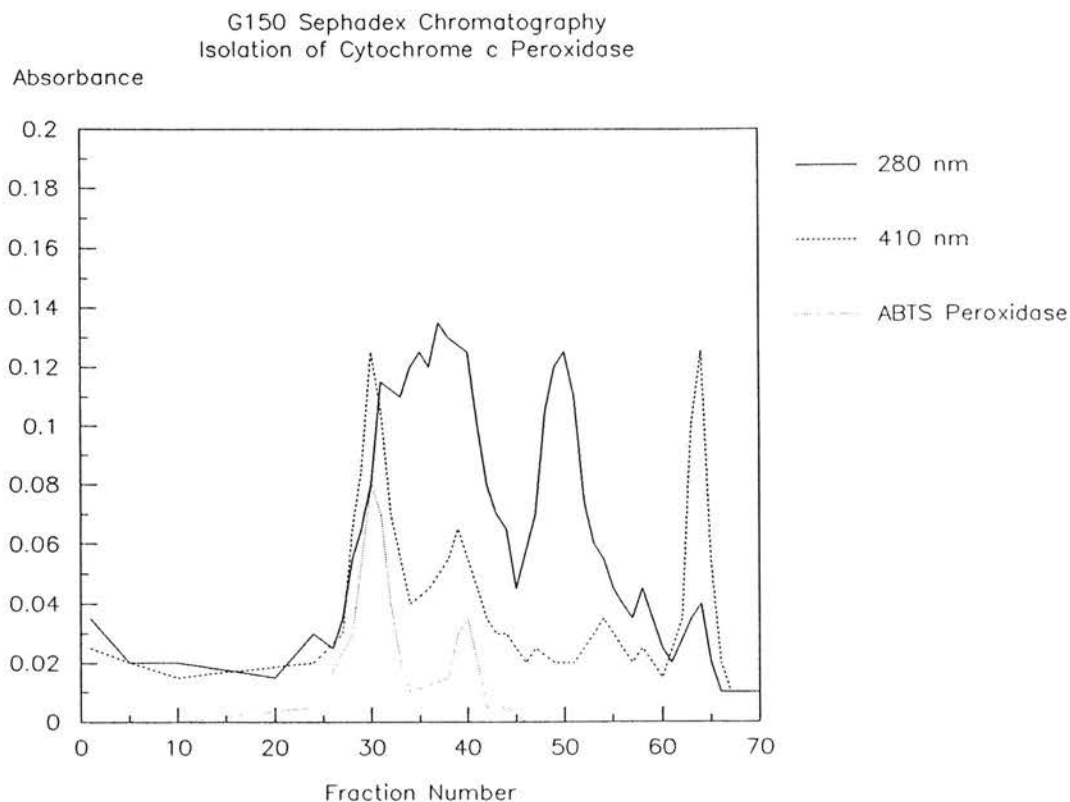


Figure 10 Purification of cytochrome-c peroxidase by G150 Sephadex chromatography. The G150 superfine Sephadex column (2 x 80cm) was packed, equilibrated and eluted with 20mM TRIS-Cl/100mM NaCl, pH8 at 4°C. *C.mucosalis* cells were grown for 1 day. 2ml of the supernatant, derived from a cell suspension containing 30mg protein per ml disrupted by freeze-thaw treatment, was applied to the column. Fractions of 3.8ml were collected and their absorbance read at 280nm and 410nm. 1ml of each fraction (or alternate fractions where the A_{410} or A_{280} was low) was assayed for ABTS peroxidase by the method described in figure 8a. 1ml of fractions 30, 40 and 50 were also assayed for cytochrome-c peroxidase activity. The cuvette contained 5uM horse cytochrome-c, 20mM Na phosphate, 1mM EDTA, 7mM TRIS-Cl, 33mM NaCl (pH7). the reaction was started by the addition of 17uM H_2O_2 . The cytochrome-c peroxidase activity recorded was negligible at 0.6, 0 and 0 nmol cytochrome-c oxidised per minute by fractions 30, 40, and 50 respectively.

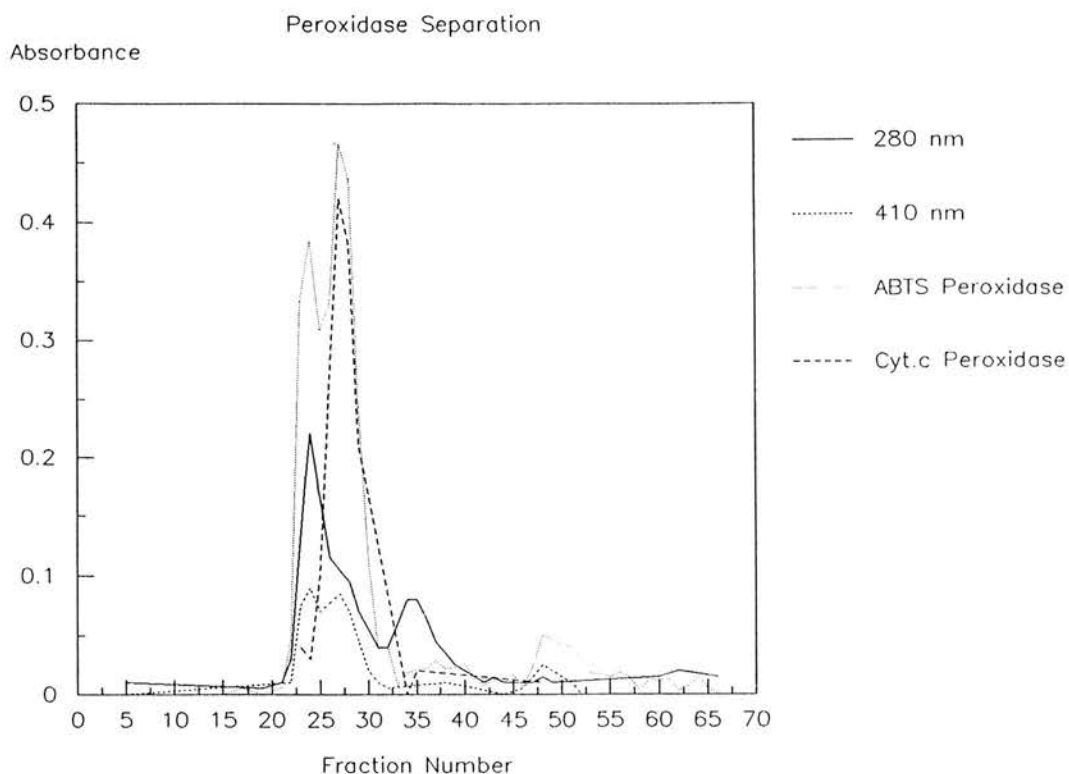


Figure 11 Purification of cytochrome-c peroxidase by G75 Sephadex chromatography. The G75 superfine Sephadex column (2 x 80cm) was packed, equilibrated and eluted with 20mM TRIS-Cl/100mM NaCl, pH8, at 4°C. The column was calibrated with the following M_r markers Blue dextran (void volume), bovine serum albumin (66 000), Ovalbumin (45 000), and horse cytochrome-c (12 000), which eluted with fractions 22, 27, 31 and 44 respectively. 1.5ml of a Freeze-thaw supernatant (derived from a cell suspension containing 30mg protein per ml) was applied to the column. Fractions of 4.5ml were collected and their absorbance read at 280nm and 410nm. 50ul from each fraction was assayed for ABTS peroxidase activity using the microwell ABTS peroxidase assay (as in figure 9b). 4ml from fractions 23 to 29 inclusive, 34, 35 and 48 were dialysed against 5mM TRIS-Cl, pH8, at 4°C for 3-4 hours and then samples were assayed for cytochrome-c peroxidase activity. The assay cuvette contained 7uM reduced horse cytochrome-c, 1mM EDTA, 5mM TRIS-Cl, pH 7.3. The activity was derived from the initial reaction rate. An absorbance value of 0.1 is equivalent to the oxidation of 1.5nmol horse cytochrome-c $\text{minute}^{-1} \text{ ml fraction}^{-1}$.

c) SDS polyacrylamide gel electrophoresis of Sephadex fractions.

Samples of Sephadex fractions from both of the ABTS peroxidase peaks, were run on SDS polyacrylamide gels, and then stained for the presence of c-type cytochromes (see chapter 5 and Goodhew et al. 1986). The major electrophoretic bands revealed (Figure 12) however were of 54 000 and 50 000 M_r and these bands were also common to Sephadex fractions both with and without cytochrome-c peroxidase activity.

Minor electrophoretic bands of 36 000 and 39 000 M_r , and therefore of similar size to the Ps.aeruginosa cytochrome-c peroxidase subunit, were seen on gels run with concentrated samples (Figure 12). These minor bands were confined to fractions possessing HHC peroxidase activity, however the level of staining was very poor, so it was possible that these bands were also present in other fractions, but below the threshold for the haem-staining method (see chapter 5).

The lack of a single band in SDS gels corresponding to HHC peroxidase activity, means that the nature of the cytochrome-c peroxidase was not determined, but several possibilities exist. The 39 000 and 36 000 electrophoretic bands in C.mucosalis may be subunits of a cytochrome-c peroxidase with high turnover rates of ferrocytochrome-c oxidation per haem-c of the enzyme. In YCCP, for example, very high turnover rates of yeast ferrocytochrome-c oxidation per mole of haematin were reported by Yonetani and Ray (1966), who compared this enzyme activity as second only to catalase. A highly active cytochrome-c peroxidase possessing a haem-c would, however, haem stain poorly on SDS gels, as staining is determined by the haem-c content of a protein (Goodhew et al. 1986). The major haem staining bands, with M_r of 54 000 and 50 000, may therefore be unrelated to cytochrome-c peroxidase activity, their presence being due to co-purification with the peroxidase.

The 54 000 and 50 000 M_r electrophoretic bands were

present in all Sephadex fractions from the void volume to the peak containing the cytochrome-c peroxidase activity. This suggested that these were possibly monomers, continually aggregating and dissociating during passage through the Sephadex column, and hence appearing in many Sephadex fractions. There was evidence, on the electrophoresis gels, of residual levels of dimer and trimer haem-c proteins remaining as polymers, even after SDS treatment (Figure 12, channels a-c).

Thus alternatively the 54 000 and 50 000 bands could be subunits of the cytochrome-c peroxidase, enzymatically active in the monomeric form, with an apparent M_r of 66 000 on Sephadex. An enzymatically inactive form may be produced, following protein aggregation, which would run near the void volume on Sephadex, but then appearing as the low M_r subunits on SDS gels.

A third possibility is that the cytochrome-c peroxidase of C.mucosalis is not a haem protein or, like YCCP, possesses a non-covalently bound haem (Yonetani and Ray 1965). In either case, haem stained gels would then be inappropriate for the M_r determination. Cytochrome-c peroxidases that are themselves devoid of a haem prosthetic group, have not been reported in the literature. Protein-staining the SDS gels of Sephadex fractions was not therefore attempted. No band of free haem was revealed at the solvent front in the haem-stained SDS gels, which tends to eliminate the possibility of the C.mucosalis cytochrome-c peroxidase containing a non-covalently bound haem.

In the absence of a purified sample, properties of the cytochrome-c peroxidase, in relation to its interaction with the e^- donor, were determined using crude, soluble extracts from C.mucosalis.

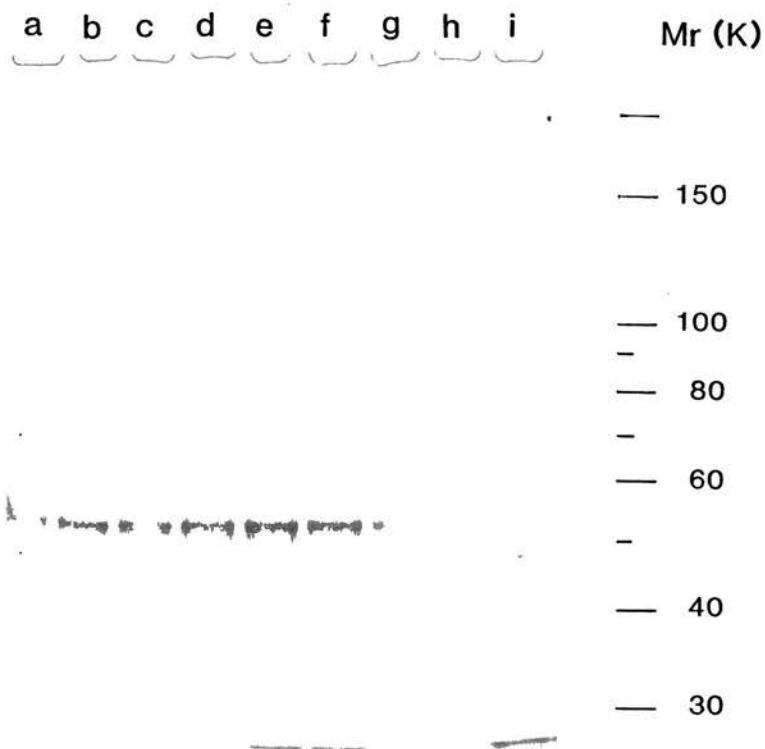


Figure 12. Haem-stained SDS-PAGE of fractions from Sephadex column used to purify cytochrome-c peroxidase. 1ml samples of fractions (which were isolated by G75 Sephadex chromatography and dialysed against 5mM TRIS-Cl as described in figure 11) were lyophilised and taken up in 80ul H₂O, thus concentrating the samples over 10 times. 20ul of these concentrated samples were treated with electrophoresis dissociation buffer then run on a 7.5% acrylamide/0.2% bisacrylamide SDS polyacrylamide gel and stained for haem peroxidase activity. Lanes a to g were fractions 23 to 29 inclusive, lane h contained fraction 34 and lane i contained fraction 48. One lane was loaded with high M_r markers:- myosin 205 000, β -galactosidase 116 000, Phosphorylase-B 97 400, bovine serum albumin 66 000, ovalbumin 45 000 and carbonic anhydrase 29 000, and then stained for protein (not shown). The relative mobilities were used to construct the scale of M_r.

5) Determination of the interaction of *C.mucosalis* cytochrome-c peroxidase with its electron donor.

The reaction between cytochrome-c peroxidase and cytochrome-c, its electron donor, requires that binding occurs, to allow e^- transfer to the peroxidase, followed by separation, to enable the oxidised cytochrome-c to return to its reductase in order to receive another electron.

The binding of peroxidases to cytochrome-c is electrostatic (Hoth and Erman 1984), and will therefore depend upon the overall charge of the two proteins, and on the ionic strength of the medium. The charge and conformation of the reaction sites is also important in determining the avidity to which binding occurs. The degree of 'fit' between the cytochrome-c peroxidase and its physiological donor is likely to be higher, than that between the peroxidase and a non-physiological donor.

The ionic strength dependence of cytochrome-c peroxidase activity was determined in *Ps.aeruginosa* by Ellfolk and co-workers, and in yeast (Kang et al. 1977, Yonetani and Ray 1966). The optimum ionic strength for *Ps.aeruginosa* peroxidase reacting with its physiological donor, cytochrome c-551, was higher than that with the non-physiological donor, yeast cytochrome-c (Soininen and Ellfolk 1972). Similarly the ionic strength optimum for YCCP was higher when reacting with yeast cytochrome-c, compared to horse heart cytochrome-c (Kang et al. 1977, Yonetani and Ray 1966). The explanation for this was that above the optimum, binding of ferrocytochrome-c was too weak, and below the optimum, binding of ferricytochrome-c was too strong (Kang et al. 1977).

With *C.mucosalis*, marked inhibition of horse heart cytochrome-c peroxidase activity occurred, following Sephadex gel exclusion chromatography in the presence of 100mM NaCl. The experimental aims therefore were:-

a) To determine the ionic strength dependence of the C.mucosalis cytochrome-c peroxidase when reacting with the non-physiological e^- donor, horse heart cytochrome-c, in comparison to the reactions with the proposed physiological e^- donor, c-553.

b) To determine the specificity of C.mucosalis cytochrome-c peroxidase for its e^- donor, by comparing the peroxidase reaction rates with different ferrocytochromes-c.

a) Ionic Strength Dependence

C.mucosalis cytochrome-c peroxidase exhibited considerable ionic strength dependence, when reacting with ferrocytochrome-c from horse heart. Any addition of salt, to the ionic strength of 1mM due to Na phosphate (retained to provide buffering capacity) inhibited the measured HHC peroxidase activity. 100mM NaCl virtually abolished all activity, to less than 5% of the measured level recorded with ionic strength of 1mM (Figure 13).

In comparison, with c-553 as e^- donor, 30% of the peroxidase activity remains in the presence of 100mM NaCl. A full profile, comparing c-553 peroxidase activity against ionic strength, could not be carried out due to the limited availability of the cytochrome-c.

The retention of a higher percentage of peroxidase activity, in the presence of 100mM NaCl, with c-553 as electron donor, is consistent with this cytochrome being a physiological donor to the C.mucosalis cytochrome-c peroxidase. Further evidence for this was obtained from reactivity experiments.

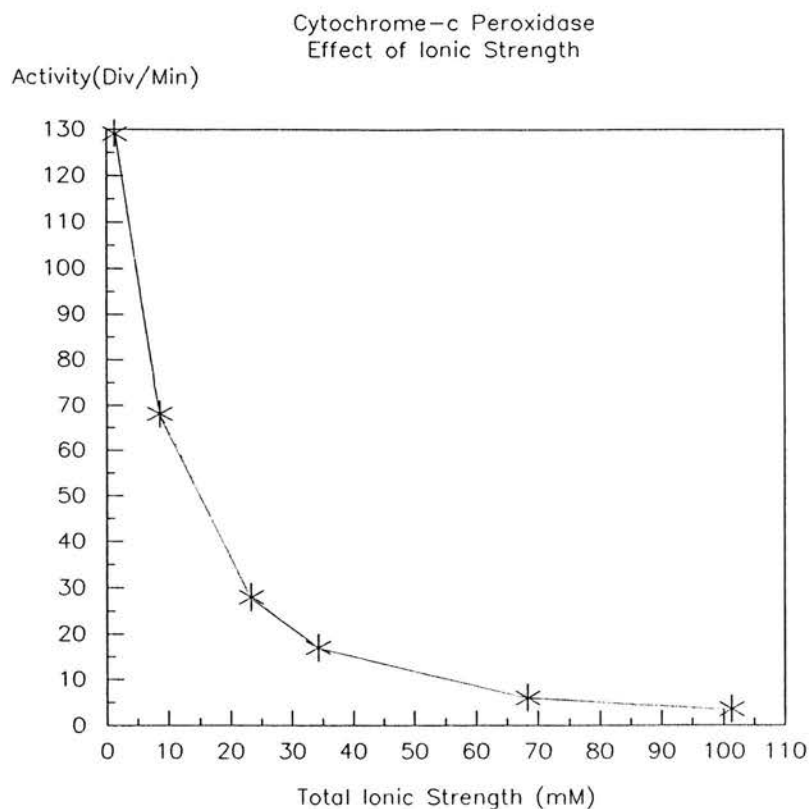


Figure 13. The ionic strength dependence of cytochrome-c peroxidase. The horse cytochrome-c peroxidase activity exhibited by 5 μ l of a freeze-thaw supernatant was determined at various ionic strengths. Ascorbate reduced and desalted horse cytochrome-c (7 μ M), 1mM EDTA and 0.6mM Na phosphate, pH7 were present. Higher ionic strengths were achieved by the addition of NaCl to the reaction mixture. The initial reaction rate was determined (expressed as divisions per minute, equivalent to 0.3nmol HHC oxidised per minute) on adding H₂O₂ to a final concentration of 17 μ M.

b) The Reactivity of C.mucosalis cytochrome-c peroxidase with its e^- donor

Quantification of C.mucosalis cytochrome-c peroxidase activity, in induction experiments (section 3 of this chapter) was carried out using ascorbate reduced desalted cytochrome-c from horse heart. This was for convenience, as this cytochrome-c was readily available. Cytochrome c-553, the proposed physiological electron donor to the C.mucosalis peroxidase, was in limited supply and was not therefore used routinely.

To test the proposal that c-553 is the physiological donor, peroxidase reaction rates with c-553 were compared to rates obtained using alternative class I type cytochromes-c as e^- donors. The cytochromes used were c-553 (from C.mucosalis), c-555 (from Chlorobium thiosulphatophilum), HHC (from horse heart) and c-551 (from Ps.stutzeri), which were all reduced in situ by titration with dithionite under anaerobic conditions. Pre-reduction with ascorbate, followed by desalting, was not an applicable method in this case, as c-553 and c-555 auto-oxidise rapidly on exposure to air, thus they become partially oxidised during passage through the desalting column.

As the activity of the cytochrome-c peroxidase itself was unstable, as described in section 2c, the assays were carried out in order of increasing reactivity of cytochrome with the enzyme. The effect of this was to compress the differences, rather than to exaggerate them.

The reactivity between peroxidase and cytochrome-c was determined by following the rate of decrease in absorbance at the α -peak, in the presence of enzyme and H_2O_2 . Pseudo-first order rate constants and initial rates, were determined as already described for HHC (section 2a).

Cytochrome c-553 was the preferred e^- donor to the C.mucosalis peroxidase (Table 4below).

TABLE 4

Peroxidase reactivity with Cytochrome c

	Net charge at pH7	Cytochrome c peroxidase			
		v(uM min ⁻¹)	% ^d	v(uM min ⁻¹)	% ^e
<u>C.mucosalis</u> c-553	+ve	20.7 ^a	100	20.8 ^c	100
"	"	16.3 ^b	79		
Horse cytochrome c	+ve	7.0 ^a	34	2.4 ^c	12
"	"	3.3 ^b	16		
<u>Chl. limicola</u> c-555	+ve			5.5 ^c	27
<u>Ps.stutzeri</u> c-551	-ve	0.3 ^a	1	0.5 ^c	2

The cytochromes were reduced in situ in a stirred cuvette under argon, by titration with 10mM dithionite (prepared anaerobically in 20mM Na phosphate, pH7). The oxidation of each cytochrome was monitored at the α -peak maximum in the presence of H₂O₂ to a final concentration of 17uM. The reaction was initiated using 25ul soluble cell extract (derived from a broken cell suspension containing 20mg protein per ml). Velocities were calculated from $v = k \times [C_r]$, where k is the pseudo-first order rate constant and [C_r] is the concentration of ferrocytochrome-c.

^a The assay was with 5.0uM ferrocytochrome-c in 10mM Na phosphate pH7/1mM EDTA.

^b The assay was as ^a, but with 3.3uM ferrocytochrome-c.

^c The assay was with 6.0uM ferrocytochrome-c in 20mM Na phosphate pH7/1mM EDTA.

^d Rates are expressed relative to 5uM c-553 (100%).

^e Rates are expressed relative to 6uM c-553 (100%).

The peroxidase rate with 5uM c-553 was 3 times higher than that with 5uM HHC. In addition, at this concentration,

c-553 was close to saturation while HHC was well below saturation. The peroxidase also reacted with the other basic cytochrome, c-555 (Table 4), but was unreactive with the acidic cytochrome c-551. The basic nature of c-553, c-555 and HHC, and the acidic nature of c-551, was demonstrated by polyacrylamide gel electrophoresis in the native state (Figure 14). Basic cytochromes are positively charged at pH7, which was the pH used during all the cytochrome-c peroxidase assays. The C.mucosalis cytochrome-c peroxidase therefore appears to have a requirement for a positively charged e^- donor, and prefers its own cytochrome c-553.

The oxidation of cytochrome c-553 by cytochrome-c oxidase, will be described in the following chapter (chapter 4). The possible roles of cytochrome-c peroxidase, cytochrome-c oxidase and cytochrome c-553, in microaerophilic respiration and also energy conservation involving H_2O_2 metabolism in C.mucosalis will be discussed in chapter 7.

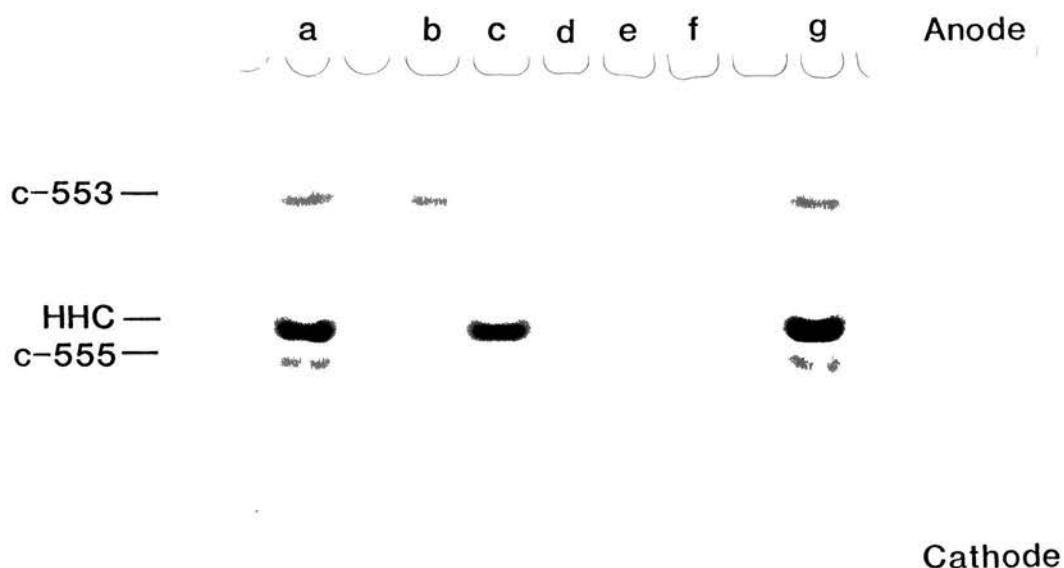


Figure 14. Native gel electrophoresis of cytochromes-c. Lanes b, c, d, e and f contained 1nmol of C.mucosalis c-553, horse c, Chl.limicola c-555, Ps.stutzeri c-551 and C.mucosalis c-553 respectively. Lanes a and g contained mixtures of all four cytochromes. After electrophoresis the gel was stained with Coomassie brilliant blue R. Ps.stutzeri cytochrome c-551 carries a net negative charge at this pH (pH8) and does not enter the gel.
HHC = Horse heart cytochrome-c.

Chapter 4

Cytochrome-c Oxidase

Bacterial terminal oxidases that are involved in the reduction of O_2 to H_2O , accept e^- from donors in the electron transport chain. Such donors are often c-type cytochromes, although other cytochromes, such as cytochrome b, can act in certain cases as e^- donors to a terminal oxidase (Poole 1983). Branched e^- transport chains to 2 or 3 terminal oxidases may be a feature of bacteria, compared to the mitochondrial system of a linear chain to a single oxidase (Pettigrew and Moore 1987).

Oxidases are identified by their characteristic visible absorption spectra, and the branched nature of bacterial systems is often shown by spectral changes following the induction of an alternative terminal oxidase. This chapter is concerned with cytochrome-c oxidase as an alternative e^- acceptor to the cytochrome-c peroxidase in Campylobacter mucosalis.

The activity of cytochrome-c oxidases, either in the whole cell system or following purification, can be measured by O_2 utilisation by the oxidase with electrons donated by artificial e^- donors such as Ascorbate-TMPD (N,N,N',N' tetramethyl-p-phenylenediamine), DMPD (N,N-dimethyl-p-phenylenediamine), DCPIP (2,6-dichlorophenol indophenol) or from cytochromes-c. The rate of oxidation of a particular cytochrome-c can indicate a possible role as physiological e^- donor to the oxidase (Froud and Anthony 1984).

The aims of the experimental work with C.mucosalis were:-

- 1) To identify the nature of the terminal oxidase or oxidases, by spectral analysis and ligand binding.

- 2) To measure cytochrome-c oxidase activity using the artificial e^- donor system of ascorbate-TMPD, and then to

study induction or repression of this ascorbate-TMPD oxidase activity under selected growth conditions.

3) To determine the specificity of the oxidase for its physiological e^- donor.

1) Spectral analysis of membranes to identify terminal oxidases.

The mitochondrial electron transport system is linear, terminating in an oxidase of the aa_3 type. Bacterial oxidases are mainly of the a_1 , aa_3 , o and d type. In addition there are cytochromes of the cd_1 type, for example in Pseudomonas aeruginosa (Wood 1978), and also non-haem copper-containing proteins in Nitrosomonas europea (Miller and Wood 1983, Rees 1968, Hooper et al. 1972) and in Pseudomonas denitrificans (Miyata and Mori 1969) that can act as cytochrome oxidases, but which are principally nitrite reductases (Yamanaka and Okunuki 1963, Miller and Wood 1983). Cytochrome oxidases have distinct visible spectral characteristics, in the reduced form and on binding carbon monoxide (CO).

<u>Oxidase</u>	<u>α-peak (red-ox)</u>	<u>(Reduced + CO) - reduced</u>
aa_3	600-607nm	591nm peak, 606nm trough
o	555-565nm	540nm + 578nm peak, 560nm trough
d	\approx 630nm peak, 650nm trough	643nm peak, 624nm trough

The aa_3 and o type oxidases are known to accept e^- from c-type cytochromes in bacteria to reduce O_2 to H_2O (Pettigrew and Moore 1987), and are therefore termed cytochrome-c oxidases.

The a_1 type, previously distinguished from the aa_3 by the presence of α -peak maxima at 585-595nm, has been found to be a variant of the aa_3 group in Nitrosomonas (Yamazaki et al. 1985). In Nitrobacter the terminal oxidase activity, which was originally ascribed to cytochrome a_1 , has more recently been attributed to cytochrome aa_3 (Pettigrew and Moore 1987). The

listing of cytochrome a_1 as a separate oxidase type is therefore of dubious validity.

The 'o' type refers to the "CO-binding pigment" of Castor and Chance 1959, which was designated cytochrome o for 'oxidase', as it was not possible to assign it by its spectral characteristics to any of the typical cytochrome groups. The 'o' type oxidase contains a b-type cytochrome, which is distinguished from other b-types by the spectral changes that occur on binding CO. The active oxidase however consists of more than 1 haem centre, possessing either cytochrome-c plus cytochrome-b types or with the cytochrome-b only (Froud and Anthony 1984). These authors therefore suggest classifying the former as cytochrome co and the latter as cytochrome bo.

The α -peak for c-type cytochromes is around 550 to 558nm and therefore there is some spectral overlap between cytochromes b (and hence o types) and cytochromes c. Some c-type cytochromes may also bind CO, to give a spectral trough at the α -peak. This has been presented as evidence for cytochromes c having oxidase activity (Hoffman and Goodman 1982, Knowles 1980, Harvey and Lascelles 1980), but CO will bind to cytochromes c that are without oxidase activity (Wood 1984).

The d type oxidase was reported not to act as a cytochrome-c oxidase (Sagi-Eisenberg and Gutman 1979a, 1979b, McInerney et al. 1984) based on the poor ascorbate-TMPD oxidase activity in bacteria containing high levels of cytochrome d (McInerney et al. 1984) and on selective inhibition of branched electron transport chains to terminal oxidases of either the d or o type (Sagi-Eisenberg and Gutman 1979a). Reports of d-type oxidases with cytochrome-c oxidase activity (Kilpatrick and Erecinska 1979) were probably due to the presence of other oxidases. The oxidation of reduced cytochrome-c by a semi-purified protozoan extract was ascribed by these authors to cytochrome-d, based on difference spectra with a peak at 615-617nm. My interpretation is that the d-type (peak circa 630nm) was contaminated with an aa_3 -type (peak at 600-607), with the latter probably being responsible for the

cytochrome-c oxidase activity observed.

No spectral evidence in the 550-650nm range was found for o, a or d type cytochromes in Campylobacter jejuni (Hoffman and Goodman 1982), or in Campylobacter fetus (Harvey and Lascelles 1980). A shoulder at 420nm in the CO difference spectra of C.jejuni (Carlone and Lascelles 1982) and in C.fetus (Lascelles and Calder 1985) was subsequently reported to indicate the presence of cytochrome o. In all these Campylobacter species, however, the major CO binding pigment was cytochrome-c.

Spectral analysis of C.mucosalis extracts (Figure 15) show mainly c-type CO binding, which was seen in supernatants and membranes, plus a small peak at 630nm with a trough at 600nm. Cytochrome b-type CO binding was not clearly seen, although this could have been masked by the high levels of c-type, and hence there was no definite evidence for the presence of an o-type oxidase. Difference spectra (Figure 16) revealed a broad peak around 600 to 640nm. The analysis did not indicate the presence of any of the 'classical' oxidases and therefore there was no spectral evidence as to the nature of a cytochrome-c oxidase. However, the absence of spectral characteristics does not preclude the presence of a terminal oxidase (Castor and Chance 1959).

Isolation of the cytochrome-c oxidase will be required to determine if a CO binding cytochrome-c is responsible for the cytochrome-c oxidase activity.

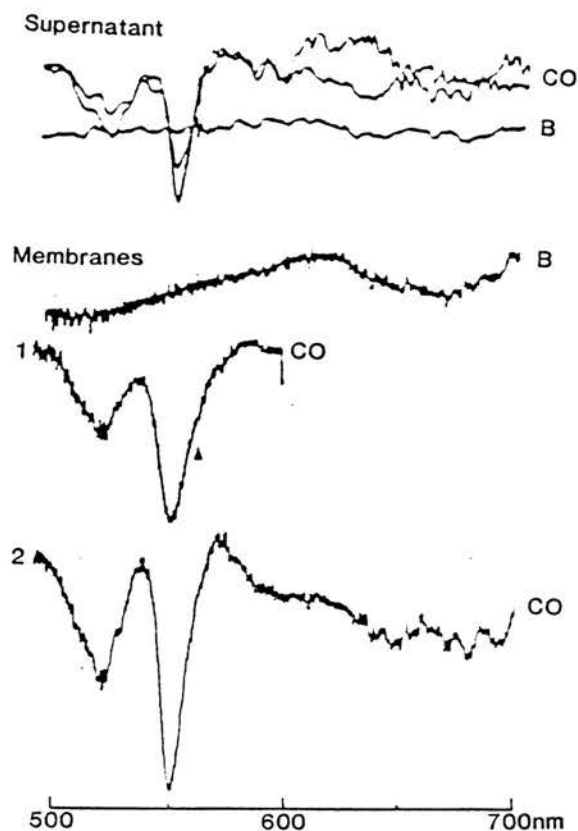


Figure 15. Carbon monoxide binding spectra of *C.mucosalis* extracts. Supernatants and membranes were from freeze-thaw extracts of *C.mucosalis*. The membranes were washed once with 10mM Na phosphate pH7 after separation from the supernatant. The samples (in 50mM Na phosphate, pH7) were reduced with dithionite and the reduced versus reduced baseline spectrum was recorded (B). Reduced + CO versus reduced spectra were made after bubbling the contents of the sample cuvette with CO gas for 15 to 30 seconds (CO). The relatively slow binding of CO to c-type cytochromes was shown by the enhanced trough at 550nm in the second of each pair of scans. The slight shoulder at 560nm (marked with an arrow) in the membrane spectrum 1 may indicate the presence of cytochrome b-type CO binding, which rapidly binds to CO.

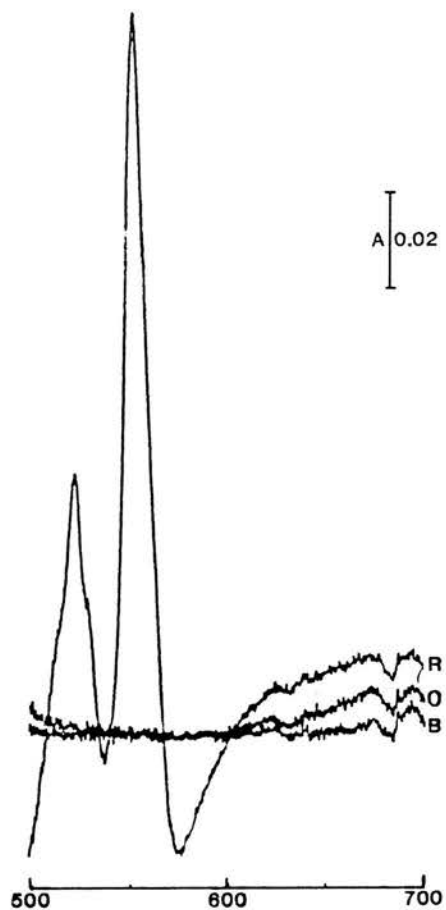


Figure 16. Difference spectra of C.mucosalis membranes. The membranes were prepared as in figure 15 and suspended in 50mM Na phosphate (pH7). The air oxidised versus air oxidised baseline was recorded (B). Air oxidised versus ferricyanide oxidised (O) and reduced versus ferricyanide oxidised (R) spectra were recorded following the addition of K ferricyanide to the reference cuvette and then dithionite to the sample cuvette respectively.

2) Cytochrome-c oxidase activity determined by ascorbate-TMPD oxidase activity.

The microbial 'Kovacs' oxidase test (Kovacs 1956) was initially used to classify bacteria with positive or negative oxidase activity, based on the presence or absence of staining with TMPD as e^- donor. The classification was subsequently further divided into 4 groups by measuring O_2 consumption with ascorbate-TMPD as the electron donor (Jurtshuk et al. 1975).

This artificial e^- donor system of ascorbate-TMPD is frequently used to determine the terminal oxidase activity of bacterial extracts. There are differences of opinion in the literature as to the mode of action and site of e^- entry to the electron transport system. This section will discuss:-

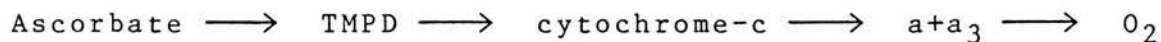
- a) The suitability of the ascorbate-TMPD oxidase assay to reflect cytochrome-c oxidase activity and the properties of ascorbate-TMPD oxidase in C.mucosalis in terms of
- b) its location within the organism,
- c) its stability
- d) its affinity for O_2 and
- e) the induction of ascorbate-TMPD oxidase activity in C.mucosalis in response to certain growth conditions.

a) The suitability of the ascorbate-TMPD system to assess cytochrome-c oxidase activity.

Cytochrome oxidase activity has been measured spectrally (Matsushita et al. 1982) by following the increase in absorbance at 520nm as TMPD was oxidised. Usually however, oxidase activity is measured by O_2 consumption in the presence of ascorbate and catalytic quantities of TMPD. The ascorbate-TMPD oxidase system is generally thought to reflect cytochrome-c oxidase activity, the e^- from the high potential TMPD ($E'_0 + 260\text{mV}$) entering the respiratory chain at the level of cytochrome-c. The rate of reduction of cytochrome-c by 10mM

ascorbate plus 0.5mM TMPD was nearly 30 times that of ascorbate alone (Kimelberg and Nicholls 1969). Ascorbate-TMPD cannot reduce O_2 directly, that is without involvement of an oxidase (Mustafa et al. 1968), although cytochrome-c modified by SDS or other anionic detergents will catalyse this reduction in the absence of an oxidase (Hill 1970).

In the mitochondrial system, TMPD donates e^- to a peripheral membrane-bound cytochrome-c :-



as only low levels of O_2 consumption were found with various cytochrome-c free mitochondrial preparations (Tyler et al. 1966) and with cytochrome-c depleted Keilin-Hartree particles (Smith et al. 1981). This low level of O_2 consumption was thought to be due to slow reduction of the oxidase in the absence of cytochrome-c (Kimelberg and Nicholls 1969). Bacterial systems which are without cytochrome-c, for example Acinetobacter lwoffii (Jones et al. 1975), are also unable to oxidise ascorbate-TMPD. TMPD oxidase activity was found to be proportional to the concentration of cytochrome-c in Azotobacter vinelandii (Jurtshuk et al. 1981). TMPD reduction of cytochrome-c occurs while the latter is complexed to the oxidase (Ferguson-Miller et al. 1978) and this tight binding does not impair e^- transfer (Erecinska et al. 1979). The e^- accepting cytochrome-c may even be a permanent part of a co oxidase complex, as in Azotobacter vinelandii (Jurtshuk et al. 1981), Methylophilus methylotrophus (Froud and Anthony 1984, Carver and Jones 1983) and Ps.aeruginosa (Matsushita et al. 1982a). Removal of the cytochrome-c component, which does not itself have ascorbate-TMPD oxidase activity (Carver and Jones 1983), from the co oxidase complex, abolishes ascorbate-TMPD oxidation (Carver and Jones 1983, Froud and Anthony 1984, Jurtshuk et al. 1981). (Damage to the cytochrome c during its purification was claimed, however, (Poole 1983) to cause the absence of oxidase activity in the Azotobacter vinelandii preparation).

Other evidence as to the suitability of the ascorbate-TMPD oxidase system to assess cytochrome-c oxidase activity, comes from the ability of a purified co oxidase to oxidise TMPD and c-type cytochromes (Matsushita et al. 1982a) and from the similar sensitivities of high potential cytochrome-c oxidation and ascorbate-TMPD oxidation to inhibition by cyanide (Carlone and Lascelles 1982). Hoffman and Goodman (1982) found that ascorbate-TMPD oxidation in C.jejuni had a different CN sensitivity from the oxidation of other substrates. This work, however, did not compare the CN sensitivity of ascorbate-TMPD oxidation with that for cytochrome-c oxidation. As CO did not inhibit the ascorbate-TMPD oxidase (Hoffman and Goodman 1982), it was suggested that oxidation was mediated by a component other than an oxidase, although the other oxidase activities measured were also relatively insensitive to CO. TMPD oxidation was inhibited in Azotobacter vinelandii by CO and CN (Jurtshuk et al. 1981).

Reports have been made of e^- from TMPD bypassing cytochrome-c and donating directly to the oxidase. In mitochondria, this cytochrome-c bypass only occurred at very high concentrations of TMPD (0.5 - 5mM) by a low affinity system, whereas with low TMPD concentrations, oxidation principally occurred via the cytochrome-c (Sagi-Eisenberg and Gutman 1979b). The same authors (Sagi-Eisenberg and Gutman 1979a) also reported that e^- from TMPD bypassed cytochromes c_4 and c_5 in Azotobacter vinelandii. This was based on finding that the ascorbate-TMPD oxidase activity was higher than the cytochrome oxidase system. However as already stated, ascorbate-TMPD can donate e^- to cytochromes-c without the requirement for dissociation of the cytochrome-c : oxidase complex, unlike in the 'natural' cytochrome-c oxidase system where dissociation of oxidised cytochrome-c from the oxidase must occur before the next molecule of reduced cytochrome-c can react (Ferguson-Miller et al. 1978). With ascorbate-TMPD the rate limiting step is either the reduction of cytochrome-c by TMPD, or the oxidation of bound

cytochrome-c. In the absence of TMPD, the dissociation of oxidised cytochrome-c from, and the association of reduced cytochrome-c to the oxidase is likely to be limiting, and therefore a slower rate of oxygen utilisation will be observed with a physiological system.

In conclusion, ascorbate-TMPD oxidase activity should reflect cytochrome-c oxidase activity, bypassing other rate limiting steps by donating e^- direct to cytochrome-c while in association with the oxidase. Any O_2 utilised due to the oxidation of endogenous substrates will be very low in comparison to utilisation due to oxidation of ascorbate-TMPD, and this will not therefore interfere with the estimation of cytochrome-c oxidase activity.

In principle therefore the ascorbate-TMPD oxidase activity can be used in experiments with C.mucosalis to reflect the cytochrome-c oxidase activity induced under different growth conditions. This was confirmed with parallel assays for ascorbate-TMPD oxidase and cytochrome-c oxidase activity. These are described in section 2e.

The properties of the ascorbate-TMPD oxidase in C.mucosalis.

As with the cytochrome-c peroxidase and other components of the electron transport system, the location of the enzyme within the organism and its function can best be determined following the formation of spheroplasts. As described in chapter 6, this was not found possible with C.mucosalis and hence membrane and soluble extracts were prepared as described for the cytochrome-c peroxidase assays (Chapter 3, section 1). The location, stability and affinity of the ascorbate-TMPD system for oxygen were determined with these freeze-thaw or french press extracts.

b) Location of Ascorbate-TMPD Oxidase within the bacterium.

Terminal cytochrome-c oxidases are in general membrane-bound enzymes which span the cytoplasmic membrane, and obtain protons for the reduction of O_2 from the cytoplasmic pool, thus contributing to the protonmotive force.

The particulate nature of cytochrome-c oxidases (Jurtshuk et al. 1981, Lascelles and Calder 1985) and ascorbate-TMPD oxidases (Carlone and Lascelles 1982, Harvey and Lascelles 1980, Jurtshuk et al. 1967) was reported in Campylobacter species (Lascelles and co-workers) and in Azotobacter vinelandii (Jurtshuk et al. 1967, 1981).

There are a few soluble enzymes, such as the nitrite reductases of Ps.denitrificans (Miyata and Mori 1969), Nitrosomonas europea (Miller and Wood 1983, Rees 1968) and of Ps.aeruginosa (Poole 1982), that can, under certain conditions, act as cytochrome oxidases, but this activity may not be physiological. Soluble cytochrome oxidases are periplasmic (Wood 1978) and so they obtain their protons from outside, without contributing to the protonmotive force (Miller and Wood 1983)

Ascorbate-TMPD oxidase activity was confined to the membrane fraction of C.mucosalis (Figure 17). The small amount of activity present in the soluble fraction was probably due to the presence of membrane vesicles, as described in section 1 of the cytochrome-c peroxidase chapter.

Location of the terminal cytochrome-c oxidase in the membrane of C.mucosalis, means that the oxidase reaction could contribute to the formation of a protonmotive force. Energy conservation in C.mucosalis will be discussed in Chapter 6.

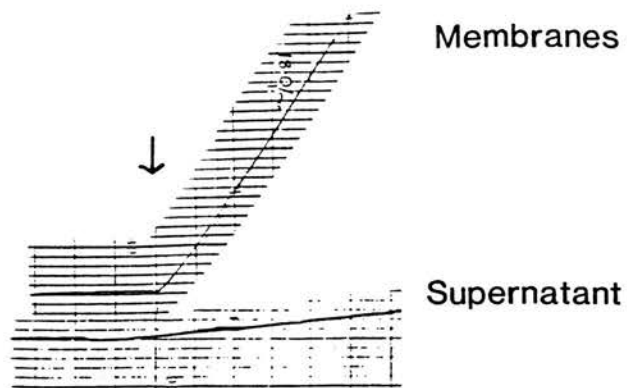


Figure 17. Ascorbate-TMPD oxidase activity in *C.mucosalis*. Oxygen utilisation was recorded in the presence of 1mM ascorbate, 0.3mM TMPD at 25°C. The reaction was initiated by adding a 50ul sample of French-press membranes or supernatant (arrow), derived from a broken cell suspension containing 20mg protein per ml. The ascorbate-TMPD oxidase activity of the supernatant was less than 10% of that found in the membrane suspensions.

c) Stability of the Ascorbate-TMPD Oxidase

Unlike the cytochrome-c peroxidase in C.mucosalis, the cytochrome-c oxidase activity was very stable when stored at -40°C . Values for O_2 consumption by the ascorbate-TMPD oxidase, after storage for 1 day, were 80-100% of the initial rate with high cytochrome-c oxidase activities still present after several weeks storage. Oxidase assays for induction experiments were nevertheless carried out using freshly prepared material from C.mucosalis.

Jurtshuk and Old (1968) reported that cytochrome-c oxidase activity was stable in Azotobacter vinelandii when stored at -15°C . However storage of Azotobacter membranes (R_3 fraction) at 4°C for 10 days led to a 3 fold increase in the specific activity of the oxidase (Jurtshuk et al. 1975), which was ascribed to the exposure of active sites (Jurtshuk and Old 1968). High oxidase activities can therefore be due to induction of activity or to the exposure of active sites with time. Exposure of active sites could occur post-mortem in cells prior to harvesting for the determination of oxidase activity. High oxidase activities in C.mucosalis grown for 3 days, as opposed to 1 day (section 2e), could be due to this effect rather than to the active induction of activity. To test this hypothesis, oxidase activities were compared after storage of a broken cell suspension for 1 day at -40°C , $+7^{\circ}\text{C}$ and $+37^{\circ}\text{C}$ (Figure 18).

Storage of broken cells at $+7^{\circ}\text{C}$ did result in a slight enhancement of ascorbate-TMPD oxidase activity to around 130% of the -40°C stored sample, which could be due to the exposure of active sites. Storage at 37°C , however, resulted in destruction of enzyme activity to around 10% of that seen in the -40°C stored suspension.

C.mucosalis cells were always grown at 37°C , therefore any increase in the oxidase activity with time was unlikely to be due to exposure of active sites, and thus can be attributed to active induction by the organism. Induction experiments will be described in section e).

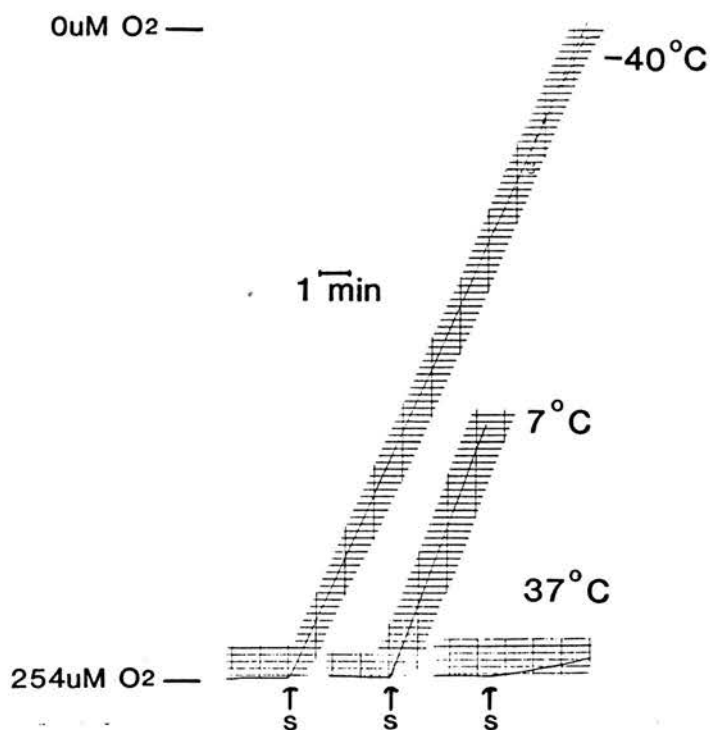


Figure 18. Effect of storage temperature on ascorbate-TMPD oxidase activity. *C.mucosalis* cells were harvested aseptically under N_2 into sterile phosphate buffered saline (P.B.S.) after 1 days growth. The cells were frozen and thawed and then sonicated with an MSE 60 Watt ultrasonic disintegrator using a sterile sonicator head for 3-5 minutes on ice. The broken cell suspension was then divided into 3 aliquots, placed in sterile bijoux and stored for 24 hours at $+37^\circ\text{C}$, $+7^\circ\text{C}$ or -40°C . 50ul samples (S) were then assayed for ascorbate-TMPD oxidase activity (conditions as in figure 17). The high affinity of the oxidase (for O_2) was shown by the linear record of O_2 utilisation from high to low O_2 concentrations.

d) Affinity of the Ascorbate-TMPD oxidase for O_2 .

Polarographic measurements of ascorbate-TMPD oxidase activity in C.mucosalis were made using an oxygen electrode (Rank Brothers, Cambridge, England). The solution in the chamber was initially saturated with atmospheric oxygen, which was 254uM O_2 at 25°C. Calibration was made to 100% with the air saturated buffer, and to 0% following the addition of the O_2 scavenger, dithionite. Over the full width of the recorder the O_2 concentration present ranges from 254uM to zero.

A bacterial sample will therefore be assayed for oxidase activity initially in the presence of a high O_2 concentration, which will gradually fall as O_2 is consumed by the oxidase reaction. A reaction trace that remains linear (zero order) at low O_2 concentrations, indicates an oxidase with a high affinity (low K_m) for oxygen.

The ascorbate-TMPD oxidase in C.mucosalis had a very high affinity for O_2 , the trace of O_2 utilisation remaining linear from 254uM to around 15uM O_2 (Figure 18). As C.mucosalis is a microaerophilic organism, adaptation to utilise low levels of O_2 by means of a high affinity oxidase is appropriate.

The high affinity ascorbate-TMPD oxidase of C.mucosalis has not been assigned to a particular 'class' of oxidase, due to the lack of any classical spectral characteristics in the membrane extracts (section 1). Isolation of the oxidase from the membranes has not been attempted for this project. The effect of growth conditions on the activity of the terminal oxidase is described below.

e) Induction of Ascorbate-TMPD and Cytochrome-c oxidase activities with Growth conditions.

The induction of oxidases with a high affinity for oxygen in response to O_2 limitation, is one aspect of the effect that growth conditions can have on terminal oxidases. In addition to microaerobic, aerobic or anaerobic conditions, cytochrome oxidases are affected by the growth phase, and by the substrates used for growth (Jurtshuk and Yang 1980). The effect of oxygen will be discussed in conjunction with the effect of growth phase, as these processes tend to change together. Other growth conditions investigated with C.mucosalis were the presence or absence of catalase in the medium and the effect of formate and hydrogen as alternative e^- donors.

Aerobic bacteria cultured in shaking flasks can obtain O_2 by diffusion from the atmosphere to replace that used for respiration. However as the cell density increases, the utilisation of oxygen will be too rapid to allow complete replacement by diffusion alone (Harrison 1973). Unless cultures are shaken and vigorously sparged with oxygen, the oxygen tension will fall during the late exponential and stationary phase of growth (Lenhoff and Kaplan 1956). Hence with microaerobic and aerobic cultures it can be difficult to separate the effects of O_2 limitation from the effects due to phase of growth. It is probable that O_2 limitation, as with limitation of a growth substrate, will cause the bacterial culture to enter the stationary phase.

O_2 limitation and the transition from exponential to stationary growth have been reported to result in the induction of alternative terminal oxidases. For example, aa_3 replaced with o (Poole 1983, Froud and Anthony 1984), o replaced with d (Bragg 1980, White 1963) and the induction of a cd_1 type (Poole 1982).

The activity of oxidases, following growth at low oxygen tensions or at different phases of growth, seems rarely to be measured. A 10 fold increase in the rate of TMPD oxidation was

noted following induction of cytochrome c (Froud and Anthony 1984), and higher NADH oxidase activities were found in Haemophilus parainfluenzae, (White 1963) in response to O₂ limitation. Cytochrome-c oxidase activity increased in Haemophilus as the culture went from the log to the stationary phase (White 1962) and in Bacillus subtilis during the passage from early to late vegetative growth (Tochikubo 1971).

C.mucosalis was grown microaerobically in sealed culture jars in which the O₂ utilised for cell respiration was not replaced and hence oxygen concentrations fell throughout growth. Cells harvested after 3 days growth were therefore exposed to lower O₂ concentrations than experienced by cells harvested after only 1 days growth.

The reliability of the ascorbate-TMPD oxidase assay to reflect cytochrome-c oxidase activities in C.mucosalis was demonstrated by comparing results obtained from assays of 1 and 3 day cell extracts (Table 5 below). 10uM HHC (pre-reduced with ascorbate and desalted prior to use) was the e⁻ donor to the cytochrome-c oxidase.

Table 5

Comparison of ascorbate-TMPD and cytochrome-c oxidase activities

	Ascorbate-TMPD oxidase nmol O ₂ min ⁻¹ mg protein ⁻¹	Cytochrome-c oxidase nmol min ⁻¹ mg protein ⁻¹
1 Day	93	94
3 Day	161	138

Comparing ascorbate-TMPD oxidase in 1 and 3 day cells grown on CBA* plates with H₂ as e⁻ donor showed that specific activities were significantly higher in the 3 day growths. 3 pairs of results were analysed using Student's t test, giving a t value of 9.2, which with n-1 (=2) degrees of freedom shows that the means were significantly different at the 98% level of confidence. (Table 6 below).

* Columbia blood agar

Table 6

The effect of culture age on ascorbate-TMPD oxidase activity.

	<u>nmol O₂ min⁻¹ mg prot⁻¹</u>	<u>Growth Conditions</u>	<u>t test</u>
1 Day	61 ^a	H ₂ + catalase	t= 9.2
3 Day	115 ^a		d.f= 2 P<0.02

^a Means from 3 pairs of assay comparisons.

Therefore ascorbate-TMPD oxidase activity was induced as the O₂ tension fell, due to cell respiration, and as the culture aged from 1 to 3 days

The growth phase was difficult to measure in C.mucosalis, but no correlation was seen between the cell yield and the oxidase specific activity. This would tend to indicate that the growth phase alone was not a major determining factor in the levels of oxidase activity measured.

The lower oxidase activity seen in the 1 day culture could be due to high O₂ levels directly repressing oxidase activity. Alternatively this effect may be related to the increased levels of H₂O₂ present under these conditions. Growth conditions that affect the production or removal of H₂O₂ were therefore used to determine if these also affected oxidase specific activities.

High levels of O₂ or the presence of formate during growth, is likely to increase H₂O₂ production (Niekus and Stouthamer 1981), whereas the inclusion of catalase in the medium will scavenge peroxide.

High O₂ levels were achieved in C.mucosalis grown with H₂ as e⁻ donor, by maintaining the O₂ concentration for 3 days at or near the initial level by daily replacement of the gaseous atmosphere in the growth jar. The ascorbate-TMPD oxidase activity (Table 7 below) of this regassed culture was slightly lower, at 82% of that found in a culture also grown for 3 days, but under conditions of continually falling O₂.

Table 7

Ascorbate-TMPD oxidase activity in 3 day cultures with and without daily oxygen replacement.

	nmol O ₂ min ⁻¹ mg protein ⁻¹
O ₂ replaced daily	116
O ₂ not replaced	142

C.mucosalis grown microaerobically for 1 day with formate as e⁻ donor, had 34% of the ascorbate-TMPD oxidase activity measured when H₂ was the e⁻ donor (Table 8 below).

Table 8

The effect of electron donor during growth on ascorbate-TMPD oxidase activity.

<u>Electron donor</u>	nmol O ₂ min ⁻¹ mg protein ⁻¹
Formate	11
Hydrogen	36

The presence of erythrocytes, which contain catalase, in the growth medium gave higher ascorbate-TMPD oxidase activities, which were 136% of those found in cells grown in the absence of erythrocytes (Table 9 below). These cultures were grown for 1 day with H₂ as the e⁻ donor and, as the plates were in the same jar, the O₂ concentrations were identical throughout growth.

Table 9

The effect of catalase, in the growth medium, on ascorbate-TMPD oxidase activity.

	nmol O ₂ min ⁻¹ mg protein ⁻¹
Serum agar	22
Blood agar	30

The observed induction of oxidase activity by low O_2 , catalase and H_2 and the repression of activity by high O_2 , absence of catalase and presence of formate may therefore be due to low and high H_2O_2 levels respectively. Apart from the possibility of oxidase damaged and inactivated by peroxide (which could incidentally also be responsible for the post-mortem inactivation seen in broken cells :- stability section c), a direct effect on oxidase activity by H_2O_2 seems unlikely.

Conditions that favour H_2O_2 production have been shown to induce cytochrome-c peroxidase activity (Chapter 3). Thus conditions that lower peroxide levels seem to repress the peroxidase activity and induce the alternative e^- transfer system to the oxidase, so utilising O_2 instead of H_2O_2 as e^- acceptor.

The possible mode of action of these alternative branches of e^- transport to H_2O_2 or H_2O as e^- acceptors will be discussed in chapter 7.

3) Reactivity of cytochrome-c oxidase with its electron donor.

As with the peroxidase cytochrome-c reaction, electrostatic interaction between cytochrome-c and cytochrome-c oxidase is important, with the reaction exhibiting a strong ionic strength dependence in mitochondria (Smith et al. 1981). A 1:1 complex was formed (Millett et al. 1983) and binding of horse heart cytochrome-c to cytochrome-c oxidase has been shown to occur at or near the haem crevice (Pande and Myer 1980), with the electrostatic stability conferred by charge pair interactions between the lysines of the cytochrome-c and the negatively charged carboxylate groups on the oxidase (Smith et al. 1981). Chemical modification of the cytochrome-c oxidase (Millett et al. 1983) or of the cytochrome-c (Ferguson-Miller et al. 1978) lowered the oxidase activity, measured by O_2 utilisation, by affecting the binding affinity of the cytochrome-c to the oxidase (Ferguson-Miller et al. 1978).

Electron donor specificity has been determined spectrophotometrically in the bacterial oxidases, by following absorbance changes as ferrocytochromes-c were oxidised. For example the soluble Nitrosomonas oxidase showed a preference for e^- from its own cytochrome c-552, which was a better e^- donor than horse heart cytochrome-c (Miller and Wood 1983). In Methylophilus methylotrophus this method was used to determine which of 2 soluble c-type cytochromes was the likely physiological donor to the oxidase (Froud and Anthony 1984). Azotobacter vinelandii oxidised horse heart cytochrome-c faster than c_4 and c_5 (Jurtshuk and Old 1968). This suggests either that c_4 and c_5 are not physiological e^- donors to the Azotobacter oxidase, or that solubilisation of these cytochromes from the membrane has resulted in damage so as to render them inoperable as e^- donors (Jurtshuk et al. 1975). It is also possible that membrane-bound c_4 will not react well with the added soluble form of the cytochrome.

The aims of the experiments with C.mucosalis were to determine the reactivity of the cytochrome-c oxidase with its e^- donor in two ways:-

- a) By O_2 utilisation.
- b) By spectrophotometric assays.

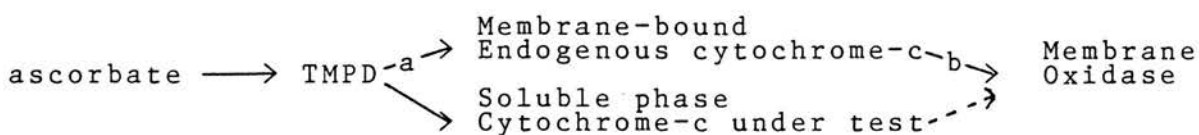
a) Reactivity with e^- donor measured by O_2 utilisation.

With mitochochondrial oxidase systems the respiratory chain preparation can be washed free of cytochrome-c using saline solutions (Tyler et al 1966) and by the preparation of Keilin-Hartree particles (Smith et al. 1981, Margoliash and co-workers). The e^- donor to be tested, for example chemically modified cytochrome-c, can then be added to mediate e^- transfer between ascorbate-TMPD and the oxidase.

Ascorbate \longrightarrow TMPD \longrightarrow cytochrome-c under test \longrightarrow Oxidase

As the rate of reduction of cytochrome-c by ascorbate is much faster in the presence of TMPD (Kimelberg and Nicholls 1969) then the oxidase activity of this system is less likely to be limited by the rate of cytochrome-c reduction, than would occur in the absence of TMPD.

In bacterial systems, such as in C.mucosalis, removal of all endogenous soluble-c is difficult to achieve (Figure 19). The presence of endogenous cytochrome-c complexed to the oxidase or the presence of a co type oxidase, would mediate rapid e^- transfer from ascorbate-TMPD to the oxidase without involvement of the cytochrome-c under test:-

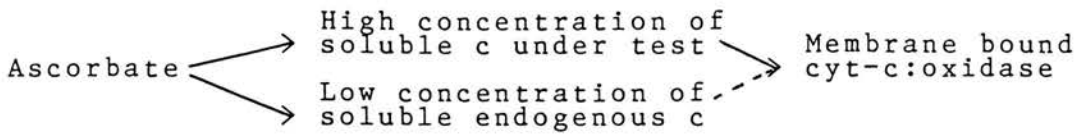


This rate of e^- transfer (a and b above) may be rapid , as dissociation of the cytochrome-c:cytochrome-c oxidase complex is not required to allow e^- transfer from TMPD (Ferguson-Miller et al. 1978).



Figure 19. Removal of endogenous soluble cytochrome-c from *C.mucosalis* membranes. A cell suspension (1 volume of cells to 4 volumes 10mM Na phosphate, pH7) was disrupted by freeze-thaw treatment, homogenised and centrifuged at 10 000 x g for 30 minutes. The supernatant was removed and the membrane pellet resuspended in the same buffer. Homogenisation and centrifugation steps were repeated to produce a total of 5 supernatants. 30ul samples of these supernatants were treated with dissociation buffer and run on a 12.5% acrylamide/0.3% bisacrylamide SDS gel. The supernatants 1 to 5 were loaded in lanes a to e respectively. The presence of c-type cytochromes washed from the membranes was revealed by staining the gel for haem peroxidase activity.
c-553 = Cytochrome c-553.

In the absence of TMPD, ascorbate reduction of cytochrome-c generally occurs while the cytochrome is dissociated from the oxidase (Ferguson-Miller et al. 1978)



and hence interference by endogenous cytochrome-c will be minimal, as this will be at a much lower concentration than the cytochrome under test.

The rates of O_2 utilisation by C.mucosalis membranes were measured with ascorbate reduced C.mucosalis c-553, Chlorobium thiosulphatophilum c-555, Ps.stutzeri c-551 and horse heart cytochrome-c as the alternative e^- donors to the oxidase. All these cytochrome are class I c-types, as described in chapter 1. Rates of O_2 consumption by C.mucosalis were considerably lower with the ascorbate-cytochrome c oxidase system, than were found in the ascorbate-TMPD oxidase system (Table 10 below).

All cytochromes-c were 10uM, ascorbate was 1mM and calibration of the reaction chamber was as for the ascorbate-TMPD oxidase assays (section 2d).

The steady-state rates of O_2 utilisation were highest with c-553 (table 10 below), with the other basic cytochromes c-555 and horse heart c also capable of donating e^- . The acidic cytochrome c-551 was unreactive with C.mucosalis oxidase.

Table 10

Effect of electron donor on oxygen utilisation by *C.mucosalis* membranes

	<u>nmol O₂ min⁻¹</u> <u>steady state</u>	<u>mg protein⁻¹</u> <u>initial rate</u>	<u>% reduced</u> <u>cytochrome-c</u>
<i>C.mucosalis</i> c-553	4.8	10.1	8
Horse heart c	3.7	5.9	49
<i>Chlorobium</i> c-555	2.7	9.1	2
<i>Pseudomonas</i> c-551	0	0	88
Ascorbate-TMPD	110	110	-

These steady state results therefore suggested that the *C.mucosalis* oxidase was most reactive with its own cytochrome c-553. The reactivity of an oxidase with its e⁻ donor depends upon its rapid association with ferrocytochrome-c, and the rapid dissociation of the ferricytochrome-c to allow another molecule of reduced cytochrome-c to bind. This assay method will only demonstrate relative reactivities with e⁻ donors, if the association or dissociation between the cytochrome-c and the oxidase is rate limiting. Other possible rate limiting steps are the oxidase reaction to reduce O₂ to H₂O, and the rate of re-reduction of oxidised cytochrome-c by ascorbate.

As the O₂ consumption in the presence of ascorbate and TMPD was very much higher than in the ascorbate-cytochrome c system, then the O₂ reduction reaction cannot be rate limiting. To avoid rate limitation, the re-reduction of the cytochrome-c by ascorbate should be sufficiently rapid to ensure that a high percentage of the soluble cytochrome-c is maintained in the reduced form and hence available as substrate to the oxidase. A high level of cytochrome-c in the oxidised form would indicate that the supply of e⁻ to the oxidase was limiting the rate of O₂ utilisation. The redox state of the cytochrome-c was estimated by transferring the contents from the O₂ electrode chamber into a cuvette and then

determining the level of reduction spectrophotometrically. c-553 and c-555 were found to be over 90% oxidised, horse cytochrome-c was approximately 50% reduced and c-551 was more than 80% reduced (Table 10 above). Therefore the rate of ascorbate reduction was limiting the observed rate of O_2 utilisation with c-553, c-555 and possibly also with horse cytochrome-c.

Ascorbate re-reduction will however not be rate limiting during the initial oxidation of reduced cytochrome-c. As 20 nmol of cytochrome-c were present and assuming full reduction by ascorbate at this concentration of ascorbate, in the absence of the oxidase, then 20 nmol of e^- were initially available to the oxidase. $4e^-$ are required to reduce each molecule of O_2 to water, thus 20 nmol of reduced cytochrome-c can donate e^- to reduce 5 nmol of O_2 . The O_2 chamber was 254 μM with respect to O_2 , in a total volume of 2ml equivalent to 508 nmol of O_2 . The initial rate of O_2 consumption, before ascorbate re-reduction became limiting, would therefore occur between 254 and approximately 252 μM O_2 , which is represented by only 1 division on the chart paper. An accurate measure of the rate of O_2 utilisation was difficult to obtain from such a trace, but the observed rate was higher during this initial period than that seen during the steady state. An estimate was made of this initial rate (Table 10 above) which again showed that c-553 was the preferred e^- donor to the oxidase. The ranking of the reactivity of the C.mucosalis oxidase with the other cytochromes-c was changed. c-555 was now seen to be preferred to horse cytochrome-c, which was not the case when ranked according to the steady-state when the rate of O_2 utilisation with c-555 as e^- donor was greatly limited by the slow ascorbate re-reduction.

The difficulties of accurately measuring these low rates of O_2 utilisation and the finding that ascorbate re-reduction was so rate limiting, meant that firm conclusions as to the reactivity of the C.mucosalis oxidase for its e^- donor could not be made on the basis of polarographic measurements.

b) Reactivity with e^- donor determined spectrophotometrically.

The reactivity of cytochrome-c oxidase with its e^- donor can be determined by following the absorbance changes as reduced cytochrome-c is oxidised, and this method has been used for bacterial systems (Froud and Anthony 1984, Miller and Wood 1983, Jurtshuk and Old 1968).

Miller and Wood (1983) and Froud and Anthony (1984) prepared cytochrome-c for the assays by pre-reducing with ascorbate and dithionite respectively, followed by desalting on G25 Sephadex to remove excess reductant. These methods could not be used with C.mucosalis experiments as the cytochromes c-553 and c-555 autooxidise quite rapidly on exposure to air. In situ dithionite reduction cannot be used to reduce the cytochromes, as it reacts with oxygen (see chapter 3). For oxidase assays, unlike the peroxidase assays, oxygen must, of course, be freely available to the system. Therefore the cytochromes-c were reduced in situ using low levels of ascorbate. As ascorbate was then present in the cuvette during the oxidase assay, then re-reduction of the cytochrome could interfere with the measurement of cytochrome oxidation. 30uM ascorbate, the concentration used to reduce horse cytochrome-c for assays of cytochrome oxidase activity in Paracoccus denitrificans (Berry and Trumpower 1985) was claimed to give negligible re-reduction of the cytochrome. With the e^- donors for the C.mucosalis cytochrome-c oxidase experiments, the rates and levels of reduction obtained with ascorbate were variable. 67uM ascorbate gave adequate levels of reduction with HHC, c-551 and c-555 (96%, 91% and 67% reduced respectively). This concentration of ascorbate however gave only 27% reduction of c-553, therefore 400uM ascorbate was used with this e^- donor, which gave 52% reduction.

The different levels and rates of ascorbate reduction obtained, made direct comparisons of oxidase specificity difficult. From table 11 (below) it appears that the preference of the C.mucosalis oxidase was for the Chlorobium

c-555 as e^- donor, rather than for the C.mucosalis c-553.

Table 11

Reactivity of the oxidase with cytochrome-c and the rate of cytochrome-c reduction by ascorbate.

	Cytochrome-c ^a Oxidase		Ascorbate reduction ^c of cytochrome-c	
	k (min ⁻¹)	% ^b	k (min ⁻¹)	% ^d
c-553	3.45	100	1.30	38
HHC	1.56	45	0.73	47
c-555	5.93	172	0.13	2
c-551	0.055	2	0.11	200

Cytochromes were 9uM in 10mM Pi pH7, 1mM EDTA.

^a Reduction of cytochromes with ascorbate, as described in ^c. The oxidase reaction was initiated with 5ul of a membrane fraction derived from a broken cell suspension containing 20mg protein per millilitre.

Pseudo first order rate constants were derived from plots of $\log (A_t - A_\infty)$ against time, where A_t is the absorbance of the α -peak at time t and A_∞ is the absorbance at the steady state of oxidation.

^b Rate constants are expressed relative to those obtained with C.mucosalis c-553 (100%).

^c The cytochromes c-551, c-555 and horse-c were reduced with 67uM ascorbate. Cytochrome c-553 was reduced with 400uM ascorbate.

Pseudo first-order rate constants were derived from plots of $\log (A_\infty - A_t)$ against time, where A_∞ is the absorbance achieved at the steady state level of cytochrome-c reduction with ascorbate.

The steady state level of ascorbate reduction as a % of full (dithionite) reduction was:- 52% (c-553), 96% (HHC), 67% (c-555) and 91% (c-551).

^d Rate constants are expressed relative to the corresponding rate constant for cytochrome-c oxidase activity.

However, the rate of ascorbate reduction was much faster for c-553 than for c-555 and therefore ascorbate re-reduction was such that the observed oxidase rate with c-553 would be considerably affected, whereas with c-555 this would be negligible.

The pseudo first-order rate constants for the oxidase reaction, in the presence of ascorbate, were derived and are given in Table 11 above. The reduction of the cytochromes by ascorbate also followed an exponential curve and these rate constants were also derived and are given in the same table. The relative negative contributions of the ascorbate reduction to the measured oxidase rates can thus be assessed. (From this table the oxidase rate with c-551 as e^- donor would appear to be greatly inhibited by ascorbate re-reduction. This was not in fact the case, as the observed oxidase rate was the same when excess ascorbate was removed, by desalting on G25 Sephadex, prior to the assay.)

Regardless of the assay method used, whether steady-state or initial-rate of O_2 utilisation, or spectrophotometric determinations, c-553 reacts well with the C.mucosalis cytochrome-c oxidase, consistent with a role as physiological e^- donor.

As with the cytochrome-c:cytochrome-c peroxidase reaction (Chapter 3) reactivity with cytochrome-c oxidase seems to require a positively charged and therefore basic cytochrome-c, as the acidic cytochrome c-551 was virtually unreactive with C.mucosalis oxidase.

The possible involvement of c-553 as e^- donor to the terminal enzymes cytochrome-c oxidase and cytochrome-c peroxidase in the microaerobic respiration of C.mucosalis will be discussed in Chapter 7.

Chapter 5

Quantification of Cytochrome c-553

Introduction

The reactivity of cytochrome c-553 with both cytochrome-c oxidase and cytochrome-c peroxidase of C.mucosalis was demonstrated in chapter 3 section 3, and chapter 4 section 5 respectively. These results were consistent with the proposed role for c-553 as physiological e^- donor to these terminal enzymes.

The aims of the experiments in this chapter were to determine the levels of soluble c-553 induced under various growth conditions and to compare these with the activities of its alternative e^- acceptors, cytochrome-c peroxidase and cytochrome-c oxidase.

Extracts of C.mucosalis were prepared by freeze-thaw or French pressure cell treatment as described in chapter 3 section 1, followed by centrifugation to remove membrane-bound material. Three methods were then used to quantify the levels of c-553; determination of the level of ascorbate reducible cytochrome-c, quantification following separation by molecular exclusion chromatography and the analysis of SDS polyacrylamide gels (SDS-PAGE). The methods were all initially assessed to determine their suitability, before being applied. The SDS-PAGE method was studied in detail, as a system with the potential to detect and quantify c-type cytochromes in general, rather than solely for the specific application of c-553 quantification.

I. ASSESSMENT OF THE QUANTIFICATION METHODS.

A) c-553 Quantification from the levels of Ascorbate-reducible cytochrome-c.

Cytochrome c-553 has a mid-point potential ($E_{m,7}$) of +99mV (Goodhew et al. 1988) and is therefore reducible by ascorbate and phenazine methosulphate (E'_0 +58 and +80mV respectively). c-553 is the only soluble high potential cytochrome-c in C.mucosalis (ElKurdi et al. 1982), therefore the degree to which the α -band was reducible with ascorbate and phenazine methosulphate (PMS) could be used to quantify the levels of this cytochrome in soluble extracts (Section II, ii.).

B) c-553 Quantification following Molecular Exclusion Chromatography

c-553 has a molecular weight (M_r) of around 8000 (Goodhew et al. 1988) and can be separated from the other c-type cytochromes of C.mucosalis by molecular exclusion chromatography on G75 superfine Sephadex. c-553 eluted from the column as a discrete peak (Figure 20) uncontaminated with other cytochromes-c, as revealed on SDS-PAGE where only one band with haem peroxidase activity appeared after staining. c-553 tends to oxidise in air (Goodhew et al. 1988) therefore the cytochromes-c in the G75 fractions were monitored by the Soret peak absorption, which is around 410nm for cytochromes-c in the oxidised form. Fractions pooled from the peak that eluted with M_r 8000, were then subjected to spectral analysis. c-553 was quantified using the mM extinction coefficient $E^{553}_{red-ox} = 15.59 \text{ mM}^{-1} \text{ cm}^{-1}$ (Elkurdi 1982). Quantification of c-553 isolated by molecular exclusion chromatography from soluble extracts will be described in section II, iii).

Sephadex Chromatography
Soluble Extracts from *C.mucosalis*

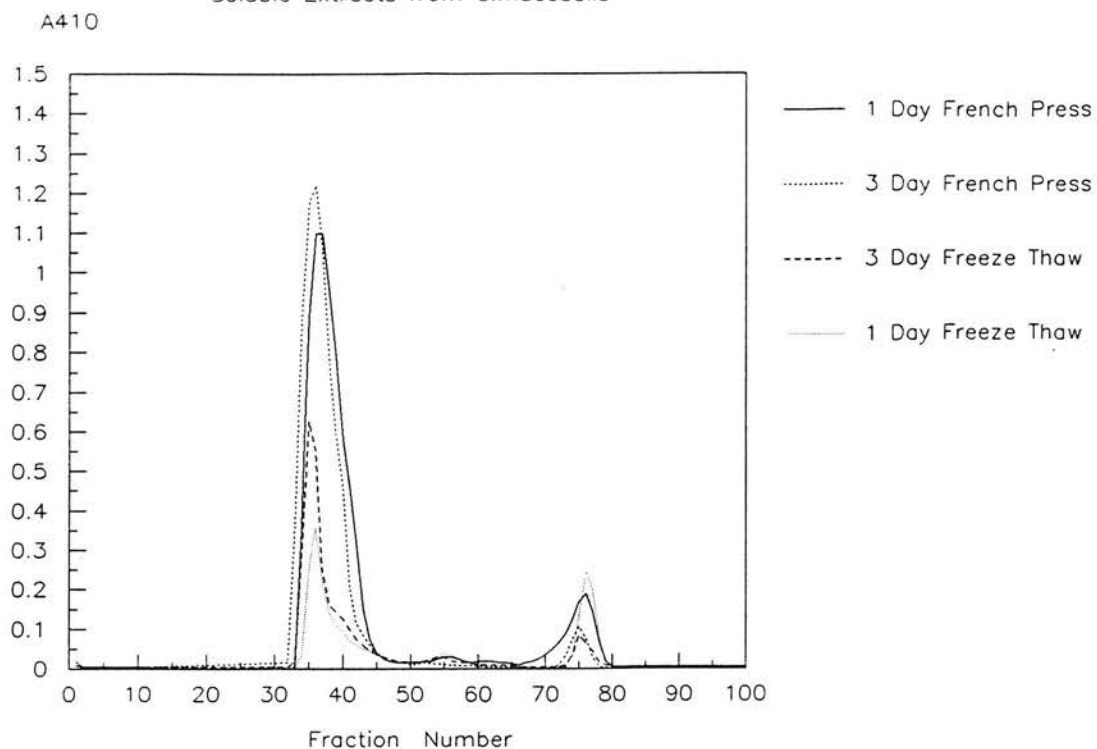


Figure 20. Molecular exclusion chromatography of soluble extracts from *C.mucosalis*. Freeze-thaw extracts from 0.56g cells, and French-press extracts from 0.44g cells, were applied to a G75 superfine Sephadex column (2 x 80cm), equilibrated and eluted with 10mM Na phosphate, pH7. The A₄₁₀ of the fractions from 1 day and 3 day extracts are shown. Cytochrome c-553 eluted as a discrete peak with fractions 70 to 80.

C) Methods for the Detection and Quantification of c-Type Cytochromes using SDS Polyacrylamide Gels

Introduction

The use of polyacrylamide gel electrophoresis (PAGE) in the presence of the anionic detergent sodium dodecyl sulphate (SDS), has been shown to separate proteins according to the molecular weight (M_r) of their polypeptide chains (Shapiro et al. 1967). M_r determinations made on continuous phosphate gels gave an accuracy of $\pm 10\%$ for polypeptides with M_r in the range of 15 to 100K (Weber and Osborn 1969). The M_r , as determined by relative mobility, was unaffected by the isoelectric point, amino acid composition or whether the proteins were globular or fibrous in the native state (Weber and Osborn 1969). With small peptides, the intrinsic charge and shape had more effect on the mobility and so in general the M_r could only be determined to within $\pm 18\%$ (Swank and Munkres 1971), and with some membrane proteins errors as high as 70% have been observed.

The temperature of solubilisation can affect the relative M_r , as a result of the degree to which the polypeptides unfold (Ward et al. 1983). With lithium dodecyl sulphate (LDS) PAGE at 0°C, migration was not according to M_r ; increasing the temperature to 22°C (SDS PAGE) resulted in migration according to M_r (Winter et al. 1980). High temperature treatment can however lead to protein aggregation (Georgiou and Webster 1987).

Sulphydryl reducing agents such as mercaptoethanol and Cleland's reagent dithiothreitol (DTT) are often included during dissociation treatment, to maintain SH groups in the reduced form, and to reduce disulphide bridges. This was necessary for dissociation of antibodies into light and heavy chains (Thomas et al. 1976) and for the complete unfolding of bovine serum albumin (Fairbanks et al. 1971). However, with the majority of proteins the presence or absence of sulphydryl reducing agents does not affect M_r determinations. This was

shown, for example, with M_r determinations of cytochrome o subunits separated on SDS PAGE (Georgiou and Webster 1987). Inclusion of thiol reducing agents is not therefore generally essential for the accurate determination of polypeptide M_r . The specific effects of sulphydryl reducing agents on the detection of haem proteins separated on denaturing gels will be discussed in sections 1, 2 and 3d.

The discontinuous SDS TRIS-Cl gel system of Laemmli (Laemmli 1970, Laemmli and Favre 1973) combines the high resolution of discontinuous electrophoresis with the ability of SDS to dissociate proteins into polypeptides. The SDS-TRIS system has been reported as superior to the SDS-Phosphate system for the determination of M_r (Weber and Osborn 1975). A modification of the Laemmli TRIS-Cl buffer system (Materials and Methods) was therefore used whenever possible, as the method of choice for the detection of cytochromes-c.

The technique of SDS-PAGE is now a standard procedure for experimental determination of M_r , after staining for protein with Coomassie brilliant blue (Weber and Osborn 1969, Laemmli 1970), Amido Black, Fast Green or Silver Staining (Pharmacia) or by the use of radio-labeled proteins detected by autoradiography (Fairbanks et al. 1965). Haem proteins can of course be visualised using these conventional protein staining methods, but there are additional methods of detection which make use of the particular properties of haem groups. Three methods which involve this prosthetic group were evaluated for use in the detection and quantification of bacterial c-type cytochromes.

- 1) Soret Peak absorbance
- 2) Fluorescent porphyrins
- 3) Haem peroxidase activity

1) Soret Peak Absorbance

In the native state, haem proteins can be detected by the absorbance of the Soret peak (circa 410nm). The mM extinction coefficient of the Soret peak is high in both the oxidised and reduced forms and is therefore more easily detected than absorbance of the α - or β -peaks, which are peculiar to the reduced form.

The absorbance at the Soret peak is frequently used to locate haem proteins following chromatographic separations and has been used to quantify certain haem proteins in solution. Polyacrylamide gels have been scanned at 410nm to detect undissociated haem-containing protein complexes, for example the b-c₁ complex (Takamiya et al. 1982), and SDS-PAGE gels were scanned to detect cytochrome f (c₁) and cytochrome b (Hurt and Hauska 1982). The latter cytochrome was detected due to residual haem retention of the non-covalently bound haem, which will tend to become dissociated from the protein during SDS treatment, with the degree of retention dependent upon the precise nature of the denaturing conditions. C-type cytochromes with their covalently linked haem, will however quantitatively retain their haem in SDS, and so the area under the peak should be proportional to the level of cytochrome present.

Reducing agents such as dithiothreitol and mercaptoethanol must be omitted as SDS treatment of c-type cytochromes in their presence leads to the removal of Fe²⁺ from the haem (Wood 1980a), which bleaches the Soret peak absorbance. Dissociation treatment in SDS in the absence of thiol reducing agents should allow retention of Fe in the covalently bound haem of cytochrome c, which can then be detected by the Soret peak absorbance following separation on SDS PAGE.

Tube gels (6 x 90mm) prepared by the continuous Phosphate/SDS system of Weber and Osborn (1969), and slab gels (1 x 130 x 150mm) prepared according to the discontinuous TRIS-Cl/SDS system of Laemmli (1970) with the addition of 2mM

EDTA, were run with different loadings of horse heart cytochrome-c (HHC), and with extracts from C.mucosalis. The gels were scanned at 410nm using a Gilford or Shimadzu CS-930 gel scanner.

The sensitivity of the 410nm scanning method to detect cytochrome-c was lower than that seen with Coomassie protein staining and it was much lower, at around 25 times less sensitive, than that achieved with the haem peroxidase method (described in section 3).

The threshold for detection of cytochrome-c on SDS PAGE slab gels with 410nm scans was 0.25nmol HHC, and the relationship between peak area and cytochrome-c was linear up to 30nmol HHC (Figure 21), which was the highest loading tested.

Theoretically it should have been possible to detect lower levels of cytochrome-c when using the thicker tube gels. However C.mucosalis extracts containing from 0.5 to 1.0 nmol total cytochrome-c were not detectable, and extracts with 1.5 to 9 nmol cytochrome-c were only poorly resolved.

Because of the low sensitivity, this method of cytochrome-c detection on SDS PAGE gels was not investigated further.

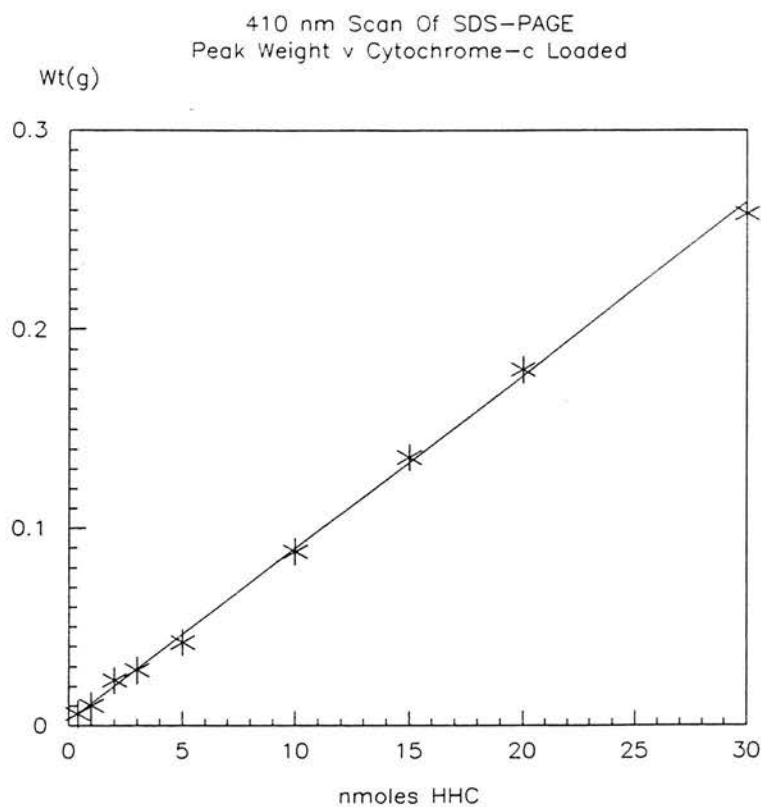


Figure 21. Quantification of cytochromes-c by their Soret peak absorbance after separation on SDS polyacrylamide gels. Samples containing 1, 5, 10, 15, 20 or 30nmol horse cytochrome-c were treated with dissociation buffer and run in a 15% acrylamide/0.4% bisacrylamide SDS gel. The centre of each lane in the unstained gel was scanned at 410nm and the area under each peak determined by cutting and weighing the chart paper. The peak weight, in grams, versus nmol cytochrome-c loaded was plotted on the graph.

2) Fluorescence of porphyrin c.

C-type cytochromes quantitatively retain their covalently bound haem in SDS, and while the iron is in the oxidised Fe^{3+} form, it tends to remain in association with the haem. Treatment with SDS in the presence of thiol reducing agents, however, leads to removal of the more readily dissociated Fe^{2+} form of iron from the haem to leave a porphyrin ring, which possesses intrinsic fluorescence when excited with near UV light (Katan 1976).

This method of detection was specific for c-type cytochromes (Katan 1976, Doherty and Gray 1979) with no other species reported to have red fluorescence (Wood 1981). Fluorescence of free haem, running at the solvent front, was reported to occur when a large loading of b-type cytochromes was applied to a gel (Katan 1976). Attempts to remove iron from the haem, to exhibit fluorescence, while retaining the non-covalently bound b-type haem in association with the protein were unsuccessful (Berry and Trumpower 1985). Therefore b-type cytochromes separated on SDS PAGE will not fluoresce in UV light.

To test the fluorescent porphyrin method, HHC standards were treated with dithiothreitol (DTT) and SDS and run on SDS PAGE gels. Tube gels were usually used, as these are thicker than slabs and this was reported to aid the detection of fluorescence (Wood 1981). The phosphate/SDS system was used which gave less variable fluorescence than the TRIS-Cl/SDS system (Wood 1981).

Gels were run in the dark to prevent photochemical damage to the porphyrin ring (Wood 1981). Dansylated proteins were run as M_r markers (Wood 1981) prepared by covalent linkage of DANS-groups to the proteins (Inouye 1971) which then fluoresce yellow in near UV light.

Fluorescence of the DANS-proteins excited at 365nm was good, but intrinsic fluorescence by the non-dansylated HHC was poor. Fluorescence was particularly poor when the dissociation treatment with SDS and DTT was carried out at 20°C rather than

at 100°C.

When the gels containing the HHC standards were viewed in daylight, instead of under UV, it was possible to see red banding. This indicated that the dissociation treatment had not resulted in the total removal of Fe^{2+} from the haem, hence formation of fluorescent porphyrins was incomplete. Various chelating agents were tested to attempt to enhance the removal of Fe^{2+} from the haem.

Phenanthroline hydrate did not improve the observed fluorescence of HHC standards, but as phenanthroline is relatively insoluble in water, it was possible that its concentration was insufficiently high to aid iron removal. The more water soluble chelator bathophenanthroline, added at a ratio of 10nmol per nmol Fe, allowed visualisation of near UV fluorescence in samples denatured at room temperature, but did not significantly improve the fluorescence of the 100°C treated standards, and was therefore subsequently omitted.

The observed detection limit, when using 1mm thick slab gels, was 1nmol cytochrome-c, therefore these were not suitable for use with bacterial extracts. These extracts contain, at the most, 50uM total cytochrome-c, consisting of four or more individual cytochromes. Hence, a 30ul sample contains around 0.4nmol of each cytochrome c, which is below the 1nmol threshold for detection on slab gels with this method.

It was just possible to detect 170pmol HHC on the thicker tube gels (Figure 22). The sensitivity was therefore much inferior to the 20pmol (Katan 1976, Wood 1980b) or 50pmol (Wood 1981) that was claimed to be detectable by this method of cytochrome-c visualisation.

Tube gels loaded with C.mucosalis extracts containing 1.6nmol soluble cytochrome-c revealed some high M_r material that fluoresced, but the small M_r c-553 was not visualised even though this is a major component of the soluble c-type cytochromes in this organism (see section II).

Lipid extraction was necessary to enable the visualisation of fluorescent bands in membrane-containing

bacterial extracts, as was reported by Wood (1981). Fluorescence was nevertheless poor, revealing only 2 cytochrome c bands of low M_r , from a loading of 1.5nmol cytochrome c. It was possible that incomplete iron removal was again the cause of the poor fluorescence. Ward et al. (1983) used more rigorous dissociation treatments, similar to the method of Jones and Jones (1970) for the preparation of demetalled cytochrome-c. This method uses the strong acid HCl to displace Fe with protons, and the weak acetic acid to chelate the displaced iron. Finally acetone and ether washes were employed by Ward et al. (1983) to remove lipid, pigment and protohaem.

Bacterial extracts treated with acid:acetone have however been found to be difficult to solubilise fully in SDS (Goodhew et al. 1986). In view of these problems with bacterial extracts, and because of the poor sensitivity, the fluorescent method of cytochrome-c detection on SDS PAGE was not applied further to the Campylobacter system. Attention was turned to the more sensitive peroxidase method for haem detection.

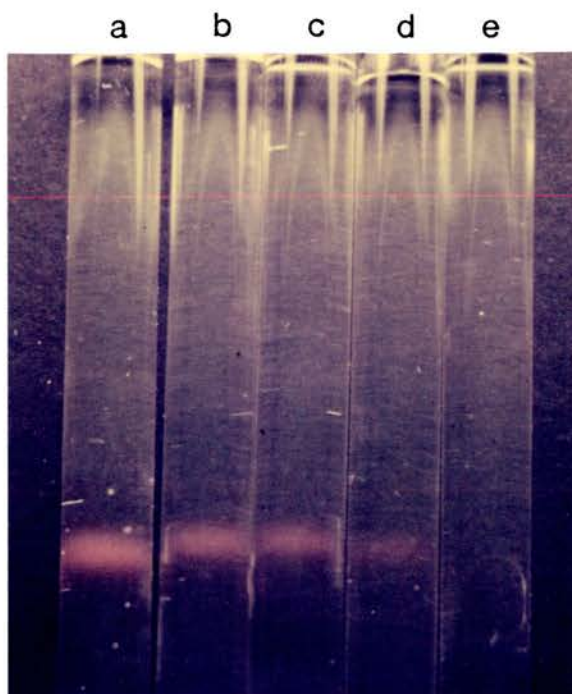


Figure 22. Fluorescence of porphyrin c. 10% acrylamide/0.3% bisacrylamide phosphate SDS tube gels (0.6 x 9cm) were prepared according to the method of Weber and Osborn (1969). The gels were pre-run with 0.1M Na phosphate/0.1% SDS as the electrophoresis buffer at 8mA per gel for 90 minutes. The samples (1.25, 0.8, 0.4, 0.17, and 0 nmol horse cytochrome-c, gels a to e respectively) were treated with 1% SDS/20mM DTT/10mM Na phosphate/15% glycerol, pH7, at 85°C for 10 minutes, cooled and loaded onto the gels. Electrophoresis was performed in the dark at 8mA per gel for 5 hours, with replacement of the electrophoresis buffer after 2.5 hours. The fluorescence of porphyrin c was viewed and photographed against a black card under near UV light (365nm) without removing the gels from their glass tubes.

3) Haem Peroxidase Activity

1) Introduction

The ability of the native form of the haem protein haemoglobin to catalyse the peroxidative oxidation of benzidine and its derivatives, has been used as an analytical method (Lijana and Williams 1979) to determine the concentration of haemoglobin in solution. Haemoglobin separated on native (non-denaturing) PAGE (Travis et al. 1970 and 1971) was stained with 3,3'-dimethoxybenzidine (DMB) and H_2O_2 . Haptoglobin (Hp) separated on starch gels (Schwantes 1976) and on native PAGE (Travis et al. 1970 and 1971) has also been detected by the peroxidase activity of haemoglobin (Hb) in the undissociated Hp-Hb complex.

Denaturation with SDS, separation on SDS-PAGE and staining with benzidine and H_2O_2 has been used more generally to detect haem proteins by the peroxidase activity of denatured haem (Welton and Aust 1974, Haugen et al 1975). Staining with benzidine was not stable and the gels became opaque making gel scanning impossible, and photography difficult (Welton and Aust 1974). The more sensitive, stable and non-carcinogenic benzidine derivative 3,3',5,5'-tetramethylbenzidine (TMBZ) was used to detect 3pmol haem on SDS-PAGE (Thomas et al. 1976).

Staining methods which use the peroxidative oxidation of benzidine or of benzidine derivatives were reported to be specific for haem proteins (Welton and Aust 1974, Haugen et al. 1975). Detergent solubilised membrane proteins from E.coli mutants, that cannot synthesise haem without δ -aminolevulinic acid supplementation, did not stain by the Thomas (1976) method after separation on agarose gels, thus demonstrating that haem was responsible for the peroxidase staining (Kranz and Gennis 1982).

Staining that was not attributable to haem peroxidase activity has however been reported for selected cases. The copper protein hemocyanin exhibited benzidine peroxidase activity, after separation on native starch gels (Manwell and

Baker 1963). The catalyst for this reaction was likely to be the copper itself, as benzidine oxidase and peroxidase activities have been ascribed to this metal (Culliford and Nickolls 1964). However, copper proteins normally lose their prosthetic group in SDS (Winter et al. 1980) and so copper proteins will not usually exhibit benzidine peroxidase activity following separation on denaturing gels. The chlorophyll in photosynthetic membranes, separated by LDS-PAGE, photooxidised TMBZ (Guikema and Sherman 1980). This photooxidation can be avoided either by staining for haem peroxidase activity in the dark (Guikema and Sherman 1980, Goodhew et al. 1986) or by prior extraction with ethanol/acetone to remove the photosynthetic pigment (Goodhew et al. 1986). Miller and Nicholas (1984) found that a Cu-containing, non-haem cytochrome oxidase/nitrite reductase from Nitrosomonas europaea used TMBZ as e^- donor for O_2 reduction, and this activity was partially retained on denaturing gels. However this staining can easily be distinguished from haem peroxidase activity by the simple procedure of viewing the TMBZ treated gel for such staining, prior to the addition of H_2O_2 . Staining due to TMBZ oxidase activity was reported to fade after the addition of peroxide (Miller and Nicholas 1984). [The distinction between DMB oxidase and DMB peroxidase activities was made in 1963 with non-denaturing starch gels (Manwell and Baker 1963). DMB treatment without H_2O_2 was used to detect the oxidase activity of ceruloplasmin, while hemocyanin was detected by its peroxidase activity following the addition of peroxide.]

Using these few precautions, benzidine-peroxidase staining can justifiably be considered to be specific for haem proteins separated on denaturing gels.

Many workers (Welton and Aust 1974, Haugen et al. 1975, Thomas et al. 1976, Guikema and Sherman 1980, Sinclair et al 1981, Takamiya and Obata 1986, Hurt and Hauska 1982, Takamiya et al. 1982, Francis and Becker 1984), rely on the residual retention of haem by b-type cytochromes following denaturation treatment, for the observed haem peroxidase staining. Black

and Bresnick (1972) demonstrated with radiolabeled haem, that SDS treatment did not completely remove the non-covalently bound haem from cytochrome P-450.

Low SDS (Francis and Becker 1984) and LDS* (Guikema and Sherman 1980, Ward et al. 1983, Sinclair et al. 1981, Takamiya and Obata 1986) concentrations and/or low temperatures (Francis and Becker 1984, Guikema and Sherman 1980, Haugan et al. 1975, Welton and Aust 1974, Sinclair et al. 1981, Takamiya and Obata 1986) have frequently been used to enhance the haem retention by b-type cytochromes and also occasionally to preserve membrane complexes (Ward et al. 1983, Winter et al. 1980). The retention of non-covalently linked haem was variable, depending upon the sample and the specific denaturing conditions used. Even using low concentrations of LDS (0.5%) and low temperatures (4°C) there was 50% loss of haem from P-450 (Sinclair et al. 1981). This high level of haem loss did not however prevent claims for the quantification of such haem proteins in TMBZ-peroxidase stained gels (Sinclair et al. 1981).

A further problem was that low concentrations of detergent have been shown to result in artifactual haem transfer to non-haem proteins (Thomas et al. 1976). Sulphydryl groups on the proteins were implicated as a probable attachment site, because the blocking agent N-ethylmaleimide (NEM) prevented the transfer (Goodhew et al. 1986).

Use of gels stained for haem peroxidase activity to detect the covalently bound and hence quantitatively retained haem of c-type cytochromes has been less extensively exploited. Ward et al. (1983) used mild dissociation treatment (0.4% LDS at 4°C) and the haem staining method of Thomas (1976) to reveal b- and c-type cytochromes. The addition of protohaem extraction led to staining of c-types only. Matsushita et al. (1982b) employed a more harsh dissociation treatment (2% SDS at 60°C) which stained for c-types only, as the addition of protohaem extraction with acid-acetone did not remove any of the haem-staining bands.

With care and the use of appropriate controls,

* Lithium dodecyl sulphate

TMBZ-peroxidase activity can be used selectively to stain for c-type cytochromes separated on denaturing polyacrylamide gels.

The aims of the experimental work were to assess the TMBZ haem-peroxidase method as a tool for the quantitative determination of c-type cytochromes and to evaluate the method for use in the specific study of bacterial cytochromes-c.

ii) Assessment of the TMBZ-Peroxidase Staining Method for Quantification of c-type cytochromes separated on denaturing SDS PAGE

The method is a catalytic process rather than stoichiometric, involving the peroxidase activity of haem exposed by SDS reacting with H_2O_2 and TMBZ. It was therefore possible that the degree of staining achieved would vary depending upon the specific origin of the cytochrome-c, and hence staining level might not be proportional to the quantity of haem loaded.

One cytochrome from a eukaryotic source (HHC) and two cytochromes-c from prokaryotic sources were used to test the method. One of the prokaryotic cytochromes was monohaem (Pseudomonas aeruginosa c-551) and the other was dihaem (P.aeruginosa c₄). All three cytochromes-c, when loaded equally according to their haem content and separated on an SDS PAGE gel, gave similar levels of haem staining (Figure 23 aged data). Therefore it was possible to quantify different types of cytochromes-c from different sources, by measuring the area under the peak.

However, it was also apparent from figure 23 that the relationship between pmol of haem loaded and the peak area was linear only from 10 to 50 pmol haem, after which a plateau of staining was seen to occur.

Factors influencing haem staining were investigated, namely a) Substrate limitation, b) Incomplete denaturation of the haem, c) Haem damage/destruction of the peroxidase centre, d) The presence of reducing agents.

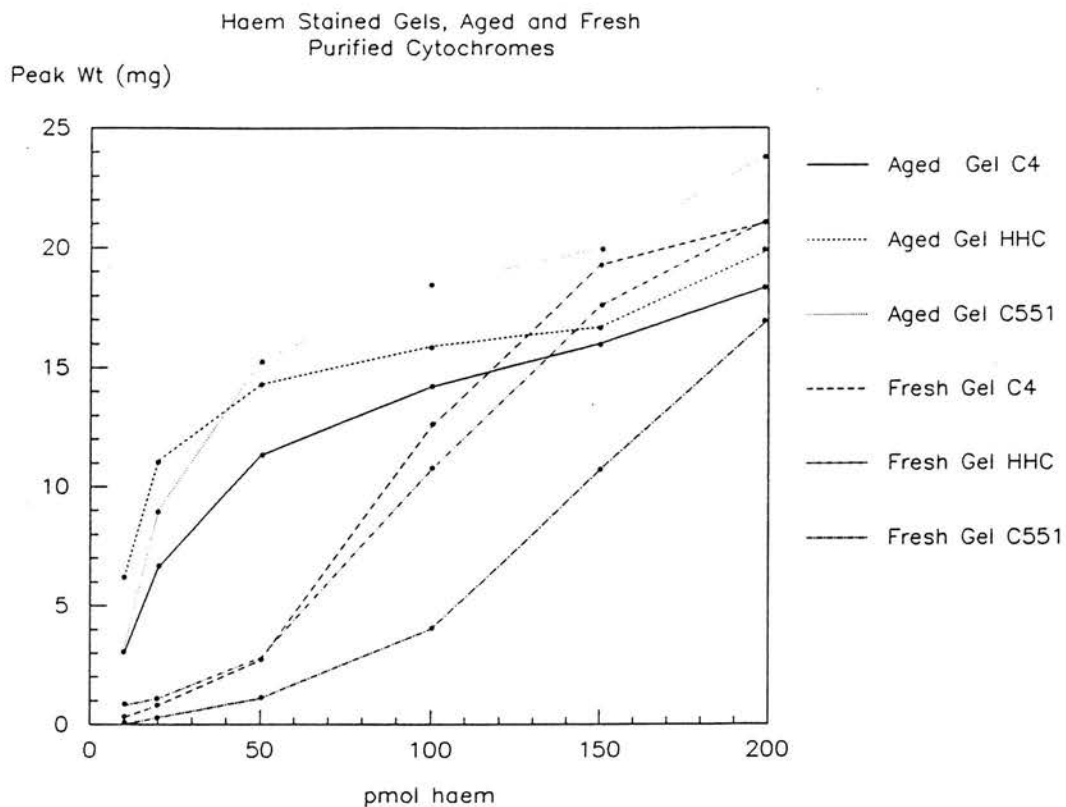


Figure 23. The variation in colour yield with quantity of purified cytochromes-c in stored and fresh gels stained for haem peroxidase activity. Electrophoresis was performed in a gel containing 15% acrylamide and 0.4% bisacrylamide. One gel was allowed to 'age' for 18 hours, the other was used freshly made. Samples contained various amounts (10, 20, 50, 100, 150, 200pmol haem) of horse cytochrome-c (HHC), *Ps.aeruginosa* c-551 and *Ps.aeruginosa* c₄. After staining for haem peroxidase activity, the central portion of each channel was scanned at 690nm and the peaks cut from the chart paper and weighed.

a) Substrate Limitation

Exhaustion of TMBZ and/or H_2O_2 were possible causes of the plateau of staining. The standard conditions used gave final concentrations of 1.25mM TMBZ and 26mM H_2O_2 . To test for substrate limitation, concentrations of 1.88mM TMBZ and/or 52mM H_2O_2 were used to stain cytochrome-c loaded with 25 to 200pmol haem. 1.88mM TMBZ (1.5 times the 'standard' conditions) was the maximum possible concentration, as this was at saturation level in solution. Increasing the concentration of either or both of the substrates did not increase the level of staining or eliminate the plateau (Figure 24). It was however still possible that local exhaustion of substrate was the cause of the plateau, but this hypothesis was not investigated further.

b) Incomplete denaturation of haem by the SDS Treatment

At high concentrations of cytochrome-c, denaturation by SDS at room temperature may be insufficient to totally expose the haem centres, hence yielding less sites for the catalytic peroxidase reaction, thus giving the plateau of staining.

Treatment with SDS at 100°C was found to be necessary with P.stutzeri extracts, but only to facilitate complete solubilisation of the sample so preventing the accumulation of high M_r polypeptide aggregates at the top of the gels. Treatment with SDS at room temperature, 37°C or at 100°C did not affect the level of staining achieved with HHC and C.mucosalis extracts.

Incomplete exposure of the catalytic haem peroxidase sites by SDS with high concentrations of cytochrome-c, did not therefore appear to be the cause of the staining plateau.

a

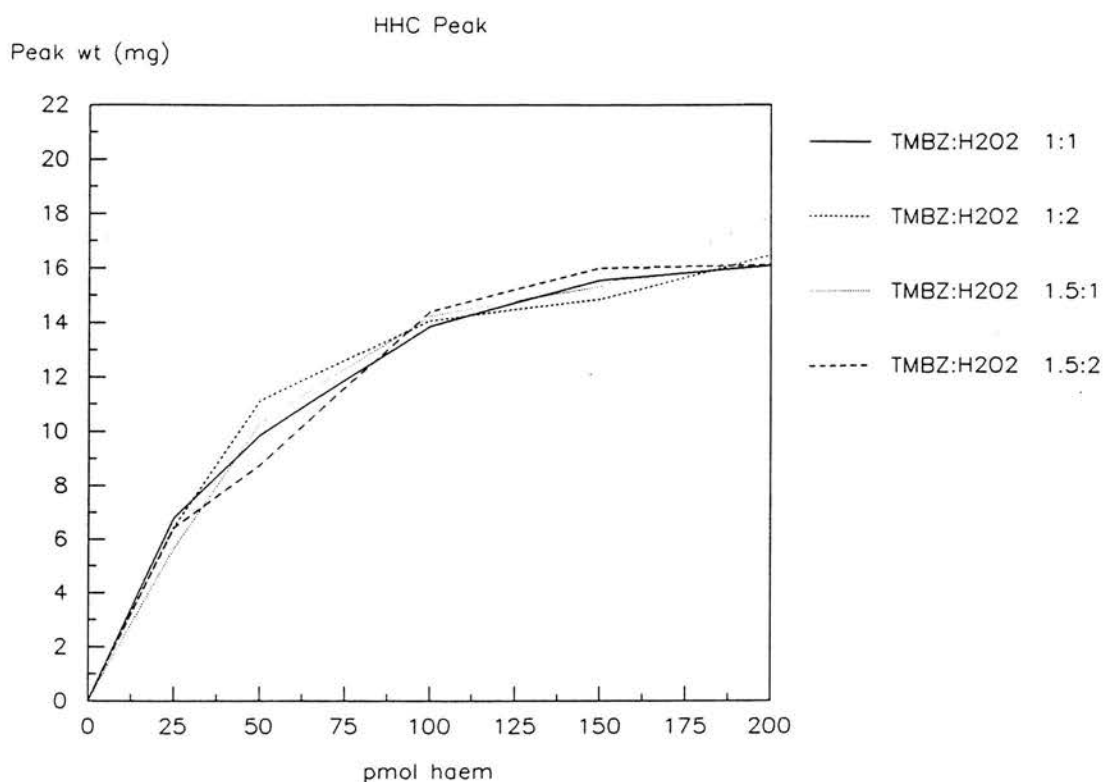
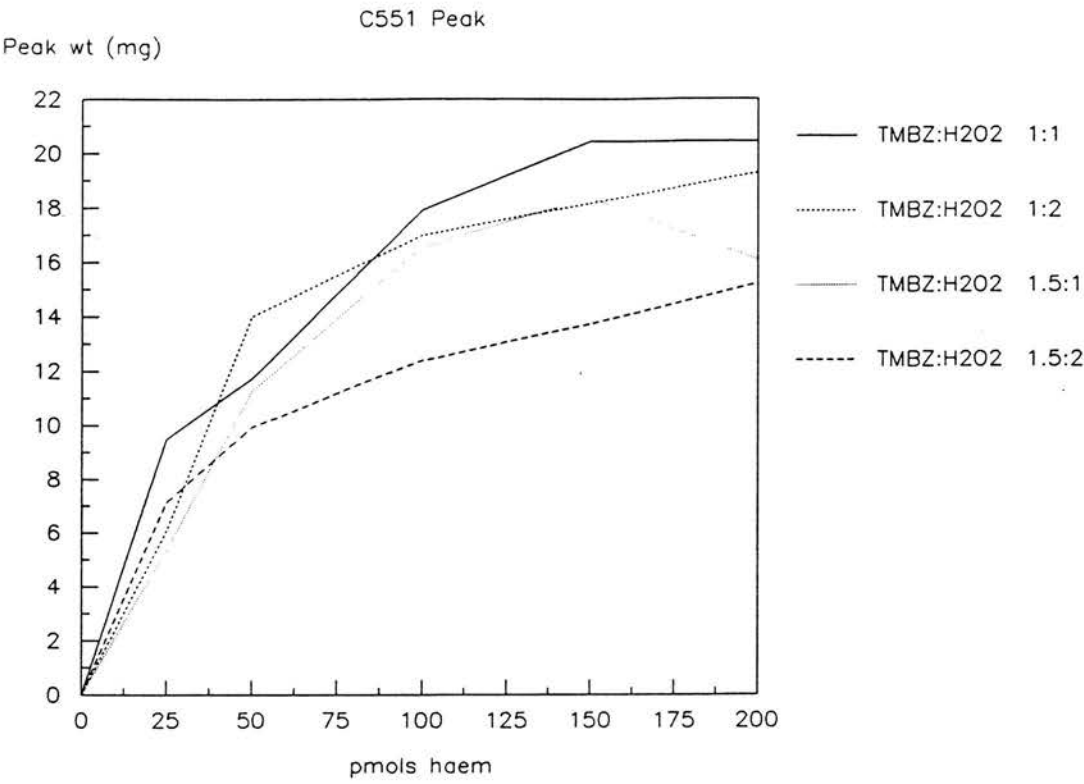
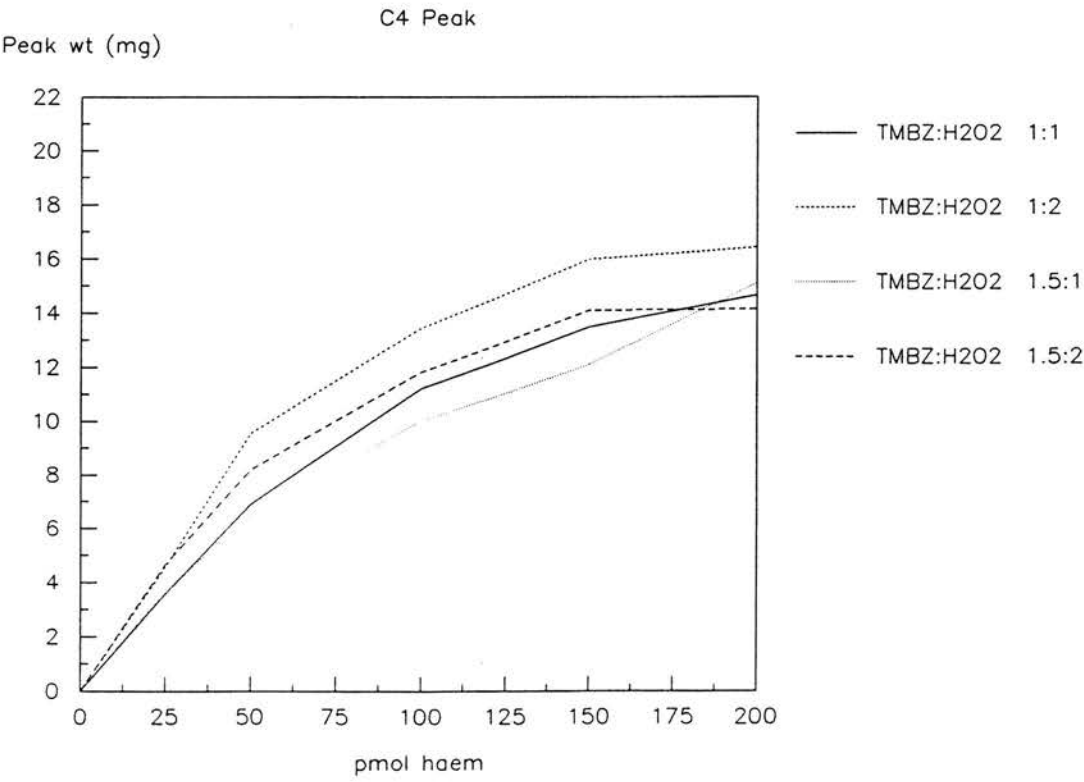


Figure 24. The effect of substrate concentrations on the colour yield obtained with gels stained for haem peroxidase activity. Electrophoresis was performed in a gel containing 15% acrylamide and 0.4% bisacrylamide on samples containing various amounts (25, 50, 100, 150, 200pmol haem) of horse cytochrome-c (HHC), *Ps.aeruginosa* c-551 and *Ps.aeruginosa* c₄. After electrophoresis the gels were stained with either:-
 1.25mM TMBZ plus 26mM H₂O₂ = 1:1
 1.25mM TMBZ plus 52mM H₂O₂ = 1:2
 1.88mM TMBZ plus 26mM H₂O₂ = 1.5:1
 or 1.88mM TMBZ plus 52mM H₂O₂ = 1.5:2
 The channels were scanned and the peak weights derived as in figure 23. The results for HHC, c-551 and c₄ are in figures a, b and c respectively.

b



c



c) Haem Damage

Haems exposed during SDS treatment are likely to be more susceptible to damage by reactive species, than are the native haems which are buried within a hydrophobic crevice.

SDS treatment will unfold and may expose regions of the polypeptide chain, that were previously inaccessible, to proteolytic enzymes. This could result in destruction of the covalent links between cytochrome-c and its haem prosthetic group, leading to loss of the catalytic site for peroxidase staining. There was little evidence for time or temperature dependent loss of haem by proteolytic activity (Figure 25). Apart from a slight effect seen with low M_r cytochrome-c, exposure to SDS for up to 7 hours did not lead to lower levels of haem staining than those observed with SDS treatment for only a few minutes prior to loading onto the electrophoretic gel. In any case, proteolysis occurring during sample preparation and the running of the gel would not be expected to adversely affect the higher concentrations of cytochrome-c more than the lower concentrations, and so this does not account for the plateau.

Inhibition of haem staining was seen when freshly prepared polyacrylamide gels were used (Figure 23) instead of gels which were 'aged' by storing for 1 day prior to use. This observation suggested that active species were present in the freshly polymerised gels, that inhibited staining directly or indirectly via damage to the haem. The free radical O_2^- superoxide, produced initially from ammonium persulphate, is necessary for the polymerisation of acrylamide (Peterson 1972), and these radicals may persist for a long time after polymerisation has occurred. Superoxide may directly inhibit peroxidation or re-reduce the peroxidatively oxidised TMBZ, thereby diminishing the staining. This phenomenon may explain the poor staining that was sometimes seen in the outer lanes of a gel. Grease on the spacer bars inhibits polymerisation of acrylamide which may result in high levels of unused, and therefore active, O_2^- radicals and hence lower levels of

staining in the adjacent cytochrome sample. The specific effects of reducing agents on haem staining will be discussed in section d).

The staining of the small, and therefore fastest running, cytochrome c-551 was inhibited the most (Figure 23). This suggested that the lower levels of haem staining was due to active species in the gel damaging the haem peroxidase site itself during the passage of the protein through the gel matrix. The c-551 running ahead of the other cytochromes, would act as a scavenger of these radicals, thereby affording some protection to cytochromes running in the rear.

Free radicals are known to destroy certain amino acids, for example tryptophan (Koziartz et al. 1978). Pre-running of gels removes persulphate, but does not prevent the damage to the proteins (Peterson 1972). Pre-running with free-radical scavengers such as thioglycollate (Koziartz et al. 1978), hydroquinone or cysteine (Peterson 1972) does however prevent this damage. However, gels cannot be pre-electrophoresed without destruction of the discontinuous buffer system. Any benefits that might have accrued from the pre-electrophoresis of continuous gel systems with free-radical scavengers, were outweighed by the loss of the superior resolution found with discontinuous gels. Co-electrophoresis of hemin with the cytochrome-c sample on discontinuous gels was investigated. The aim was to use the high mobility haem to scavenge the damaging species from the gel in advance of the cytochrome-c sample. The level of cytochrome-c staining was only slightly enhanced when hemin was present during the running of aged gels, and the plateau of staining was still seen to occur.

Gels were however subsequently always aged before use in all routine separations, to allow persulphate to decay and prevent the very poor staining seen with the fresh gels.

Haem damage, that destroyed the peroxidase centre resulting in lower levels of staining, may occur during the staining procedure itself. Inactivation by the substrate peroxide could occur in a reaction similar to the inactivation of Pseudomonas cytochrome-c peroxidase with H_2O_2 (Ronnberg et

al. 1981). Such a reaction would probably, however, affect the low loadings of cytochrome-c as much as or more than the high loadings, and would not result in the observed effect, namely the plateau of staining.

In conclusion this plateau of staining with loadings of cytochrome-c above 50 to 100pmol haem could not be eliminated. Therefore quantification of levels of cytochrome-c should be carried out in the linear region (Figure 23) from 10 to 50pmol, thereby ensuring that colour yields are below the plateau region.

This can be demonstrated in a gel with dilutions of a periplasmic extract from aerobic and denitrifying P.stutzeri (Figure 26). In all bands the colour yield was below the plateau region seen in fig 23, as the ordinates are comparable. Thus the effect of the growth conditions on the levels of cytochrome cd_1 and of the 30K cytochrome-c can clearly be seen at all loadings used.

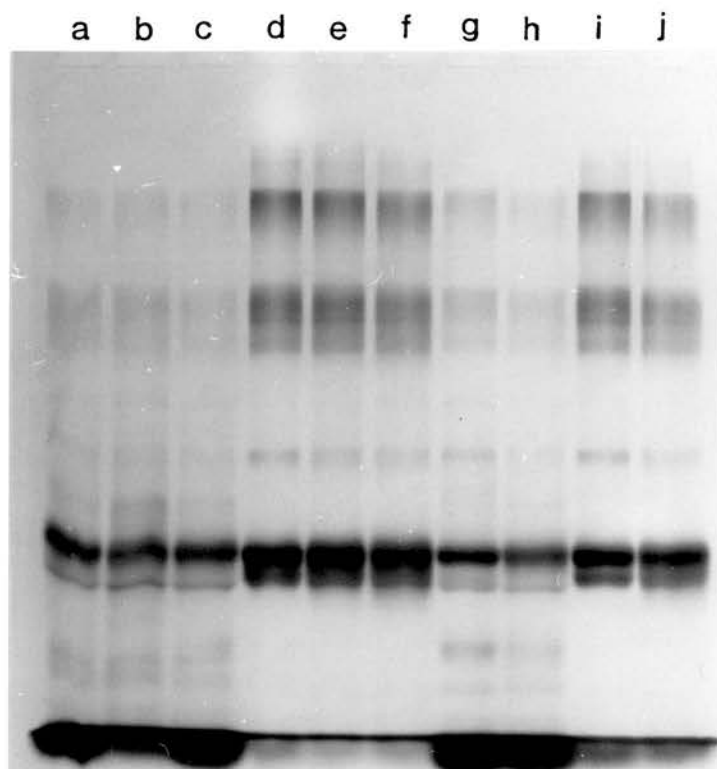


Figure 25. Effect of prolonged exposure to SDS on the colour yield obtained in haem-stained gels. Electrophoresis was in a 7.5% acrylamide/0.2% bisacrylamide gel. Samples were freeze-thaw supernatants from 1 day (Lanes a, b, c, g and h) or 3 day (Lanes d, e, f, i and j) grown *C.mucosalis* cells. The extracts were treated with the dissociation buffer for 7 hours at room temperature (Lanes a and d), 7 hours at 37°C (Lanes b and e) or 7 hours at room temperature after treatment at 100°C for 2 minutes (Lanes c and f). Samples g, h, i and j were treated just before loading onto the gel with dissociation buffer at room temperature (Lanes g and i) or at 100°C for 2 minutes (Lanes h and j). All lanes were loaded with samples (30ul) containing 0.29nmol cytochrome-c.

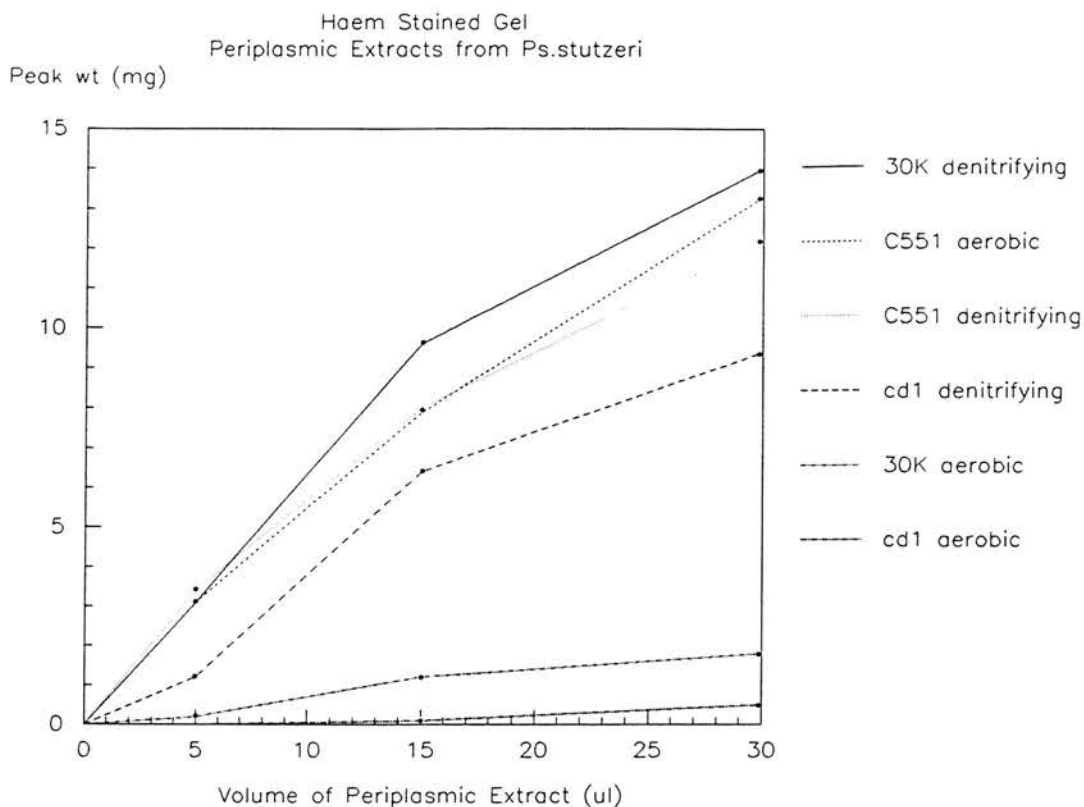


Figure 26. The variation in colour yield with the quantity of periplasmic extract from *Ps.stutzeri* stained for haem peroxidase activity. Electrophoresis was performed in a gradient gel of 10% acrylamide, 0.2% bisacrylamide to 25% acrylamide, 0.5% bisacrylamide on samples (5, 15, 30ul) of periplasmic extracts of *Ps.stutzeri*, grown under aerobic and denitrifying conditions. Periplasmic extracts were prepared by the method of Wood (1978) except that cells were suspended to a final concentration of 100mg per ml. The individual cytochromes (cd_1 , 30K and c-551) were identified by reference to purified markers. The colour yield for each band was determined as described in figure 23, and is expressed as peak weight versus ul of periplasmic extract loaded.

d) Effect of Reducing Agents

- Experimental

In section 3 ii) it was shown that, under controlled conditions, the TMBZ haem peroxidase staining was quantitatively related to the level of pure cytochrome-c applied to the gel. In early experiments with bacterial extracts of C.mucosalis it was noticed, however, that treatment with the thiol reducing agent mercaptoethanol in the presence of SDS, resulted in high losses in the level of haem staining achieved on denaturing gels. The aim of the following experiments was to quantify the effects of reducing agents on the TMBZ haem peroxidase staining activity of cytochromes-c from bacterial extracts separated on SDS PAGE.

Reducing agents from endogenous and exogenous sources were investigated. The exogenous reducing agents ascorbate, or the thiol reducing agents DTT and mercaptoethanol were used. Endogenous reducing agents can be removed by the preparation of acetone powders, ethanol-acetone or acid-acetone extraction which will precipitate the proteins, leaving the small molecular weight reducing agents in solution. However, solubilisation of the protein precipitate in SDS can be incomplete thereby leading to decreased levels of haem staining (Goodhew et al. 1986). As a practical alternative, endogenous reducing agents were removed from the protein extracts by dialysis. The sample used for the experiments was a soluble extract of P.aeruginosa grown under denitrifying conditions. The dialysis time was kept short (under 2 hours), to minimise loss of the small M_r cytochrome c-551 through the walls of the dialysis tubing.

Treatment with exogenous reducing agents was performed on the dialysed extract, to eliminate contaminating effects of any endogenous reductants. Ascorbate or DTT were added to a final concentration of 3.7mM in the dissociation buffer. A sample of the extract was also treated with DTT followed by dialysis, to remove the reducing agent, prior to dissociation

in SDS. Adjustments were made for any dilutions to the sample that occurred during dialysis treatment, by spectral determination of the cytochrome-c content.

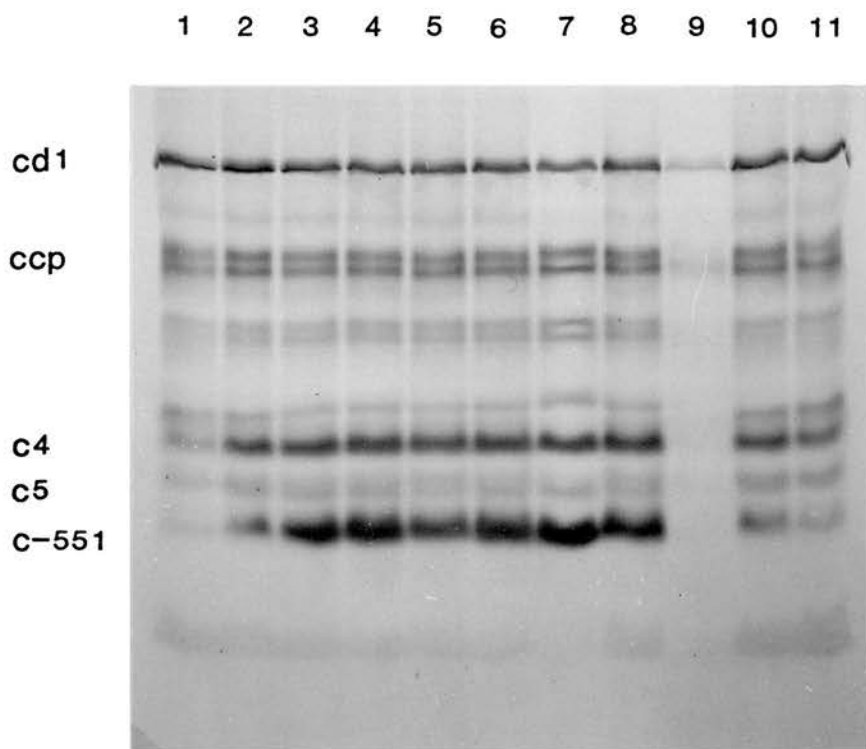
After dissociation treatment in SDS at 100°C, the samples were separated on a 15% acrylamide SDS gel. The gel was stained for haem peroxidase activity with TMBZ (Figure 27a) and the peak areas derived from a gel scan at 650nm (Figure 27b).

Pre-reduction of the sample with DTT plus dialysis prior to SDS treatment, resulted in minimal losses in the level of haem staining (Channel 11). Loss of staining was mainly seen with the low M_r cytochrome c-551, which was probably due to losses of the cytochrome during the dialysis treatment, rather than to inhibition of staining by the DTT treatment itself.

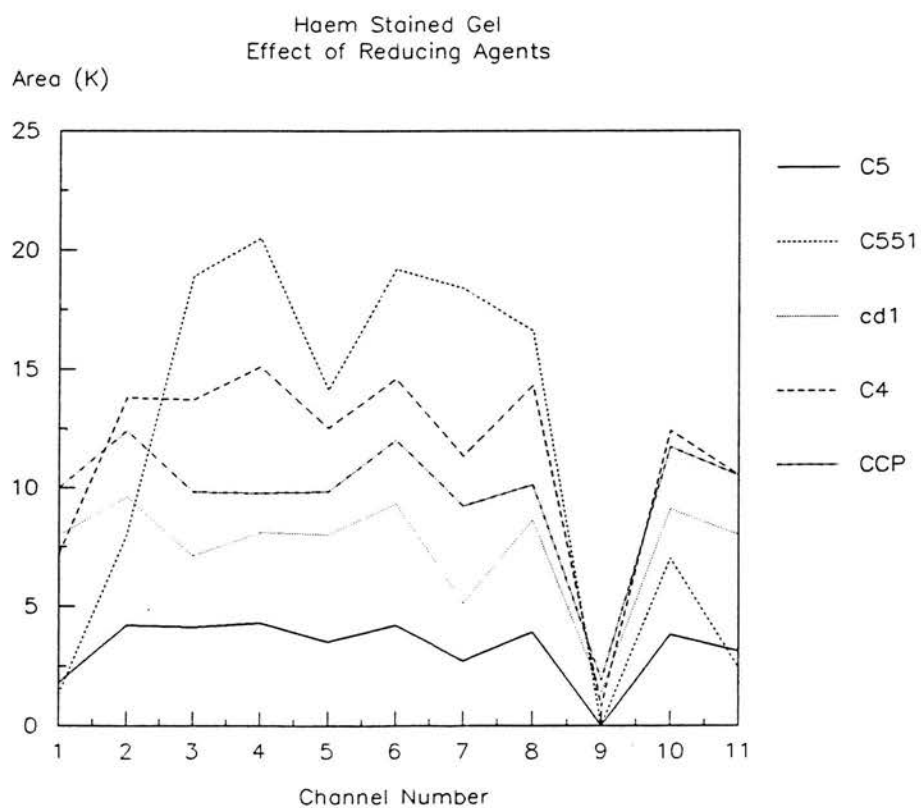
Treatment of the sample with SDS while in the presence of the reducing agent DTT did however markedly affect haem staining (Channel 9). This treatment virtually eliminated haem staining, to a level of less than 10% of that seen in the non-reduced control.

Dissociation treatment with SDS in the presence of ascorbate (which is not a thiol reducing agent), or with endogenous reducing agents, had little or no effect on the levels of haem staining achieved in P.aeruginosa (Figure 27 Channel 7 and 3 respectively). In C.mucosalis extracts, endogenous reducing agents were possibly responsible for inhibition of haem staining, as the level of staining was sometimes enhanced following acid:acetone treatment.

Figure 27. The effect of reducing agents on the colour yield obtained with a haem-stained gel of Ps aeruginosa extracts. Electrophoresis was performed on a gel containing 15% acrylamide and 0.4% bisacrylamide with French-press supernatants from Ps.aeruginosa. After treatment with reducing agents, the samples were equalised for cytochrome-c content before dissociation buffer treatment (100°C for 2-3 minutes) and loading on the gel. The supernatant was dialysed (versus 10mM TRIS-Cl pH8 at 4°C) to remove endogenous reductants, except for channel 3 which was loaded with a non-dialysed sample. Channels 1, 2, 4, 5, 6, 8 and 10 contained supernatant without further reduction treatments. Channel 7 was treated with ascorbate, and channel 9 with DTT to final concentrations (after addition of the dissociation buffer) of 3.7mM. Channel 11 was treated as channel 9, except that the DTT was removed by dialysis (in 10mM TRIS-Cl pH8 at 4°C for 2 hours) prior to the dissociation treatment. The gel was haem-stained and photographed (figure 27a) and scanned at 650nm. The areas of the c_5 , c_{551} , cd_1 , c_4 and cytochrome-c peroxidase peaks were derived from the gel scanner integration (figure 27b). CCP = Cytochrome-c peroxidase.



27a



27b

- Discussion

Thiol reducing agents were excluded from denaturing gels (Thomas et al. 1976, Guikema and Sherman 1980, Sinclair et al. 1981) as they were thought to result in loss of peroxidase staining by aiding the dissociation of haem from b-type cytochromes. The bands in the P.aeruginosa sample, that exhibited haem staining activity (figure 27b), are all well characterised as c-type cytochromes. The observed loss of staining that was seen with SDS treatment in the presence of thiol reducing agents was not therefore due to loss of non-covalently bound haem.

Thiol reducing agents were thought to destroy the centre with peroxidase activity (Berry and Trumpower 1985). Metal ion catalysed oxidation of DTT produces H_2O_2 (Trotta et al. 1974) which could, during the several hours taken to run a gel, damage the haem of c-type cytochromes. Poor haem staining in SDS gels was attributed to coupled oxidation destroying the haem ring (Francis and Becker 1984). Such oxidative haem cleavage has been shown to occur in native haemoglobin solutions in the presence of certain reductants, for example ascorbate (O'Carra 1975), but this mechanism has not been demonstrated to occur in the presence of SDS. The experimental evidence of figures 27a and 27b showed that treatment with ascorbate in the presence of SDS did not lead to the destruction of the haem peroxidase centre.

A more likely cause of the loss of peroxidase activity is that thiol reducing agents removed the iron from the SDS-exposed haem. The removal of Fe^{2+} from haem by SDS and mercaptoethanol treatment, forms the basis of the fluorescent method of cytochrome-c detection (Wood 1981, Katan 1976), see section 2. According to this interpretation, the lack of inhibition of staining seen with the reducing agent ascorbate at first sight appears anomalous (Figure 27b channel 7). Ascorbate, however, has a relatively high redox potential (+58mV), compared to the low potential of DTT (-330mV). Thus the iron in SDS denatured cytochrome-c is likely to be

reducible by DTT, but not by ascorbate. Only iron in the reduced Fe^{2+} form, can be inserted or removed from the haem, hence treatment with DTT will be much more effective at iron removal than ascorbate.

Whatever the specific cause of the loss in haem staining by treatment with thiol reducing agents, it was clear from the experimental results that these should be avoided for the preparation of samples for SDS PAGE.

Conclusions

With the use of certain precautions, the haem-staining method is an invaluable tool in the study of bacterial c-type cytochromes. This technique was used extensively during the project to assess spheroplast formation (chapter 6), to identify c-types cytochromes in chromatographic separations (Chapter 3) and to demonstrate the induction of cytochrome c-553 under varying growth conditions (Section II, below). Gel photographs were used to reveal qualitative differences in the levels of c-553 present in the extracts. Quantitative determinations were made from gel scans by measuring the area under the peak.

II. APPLICATION OF QUANTIFICATION METHODS TO DETERMINE THE LEVELS OF c-553 INDUCED IN *C.mucosalis* IN RESPONSE TO GROWTH CONDITIONS.

Two aspects of growth conditions were investigated:-

A) Effect of O₂ concentration.

B) Effect of catalase.

A) Effect of falling O₂ concentration during growth, or the effect of age of cells

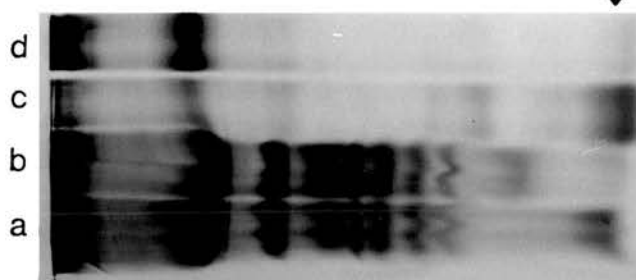
As described in Chapter 3 section 3a, the oxygen concentration falls gradually during growth. Thus *C.mucosalis* cells harvested after 3 days growth will have been exposed to lower O₂ tensions than experienced by cells harvested after only 1 days growth.

For these experiments, H₂ was used as the e⁻ donor. The cells were usually grown in the presence of erythrocytes (and therefore catalase) on Columbia blood agar, although some experiments were performed using serum agar and hence in the absence of this enzyme.

i) Quantification of c-553 levels using SDS-PAGE stained for haem peroxidase activity.

Figure 28a shows an SDS-PAGE gel loaded with 1 and 3 day *C.mucosalis* freeze-thaw extracts, stained for haem peroxidase activity. Samples (15ul), of the soluble and membrane fractions derived from 1 and 3 day cell suspensions containing 19.8 and 19.4 mg protein ml⁻¹ respectively, were applied to the gel. The gel photograph demonstrated qualitatively the presence of higher levels of c-553 in the extract from the 1 day growth. c-553 levels were quantified by scanning the gel at 650nm (Figure 28b) and weighing the peaks to determine the relative peak areas. The peak weights so derived are in table 12 below. [Preliminary tests showed by weighing that the paper was of a uniform thickness.]

28a

c-553
↓

28b

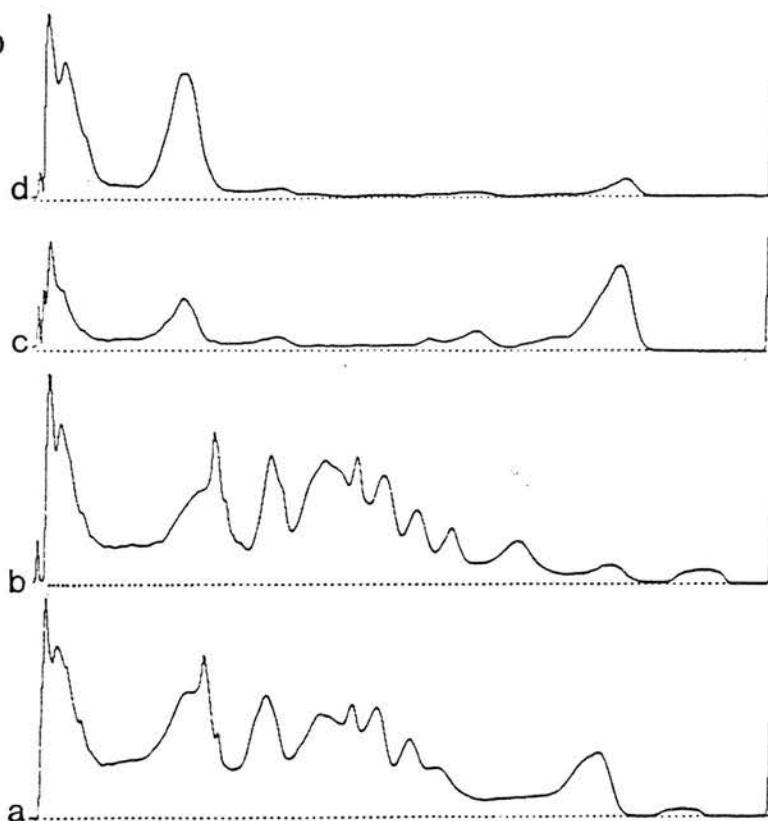


Figure 28. Quantification of *C.mucosalis* cytochrome c-553 using SDS polyacrylamide gels stained for haem peroxidase activity. Electrophoresis was performed in a gel containing 15% acrylamide and 0.4% bisacrylamide. 1 and 3 day cells were disrupted by Freeze-thaw treatment and samples (15ul) of supernatants and membranes (derived from broken cell suspensions containing 20mg protein per ml) were applied to the gel. Lanes a and b are 1 and 3 day membranes respectively. Lanes c and d are 1 and 3 day supernatants respectively. After staining, the gel was photographed (figure 28a) and scanned at 650nm (figure 28b). The position of cytochrome c-553 is marked by an arrow.

Table 12

c-553 quantification from scan of haem-stained gel.

<u>Extract</u>	<u>Weight of c-553 peak (mg)</u>	
	<u>1 day</u>	<u>3 day</u>
Supernatant	10.2	2.1
Membranes	9.0	2.4
Total	19.2	4.5

The membrane extract consisted of membrane-bound material and soluble proteins trapped within the membrane pellet and within whole cells that remained unbroken during the extraction procedure. In comparison with the total cell breakage seen after French press treatment (Figure 3), some C.mucosalis cells resisted disruption by the milder freeze-thaw method. Use of the freeze-thaw method to release soluble material (as in the above table), could therefore have affected the observed distribution of c-553 between the membrane and soluble fractions, by increasing the quantity of soluble material trapped within unbroken cells. However, comparisons of 1 and 3 day growths showed that levels of c-553 were over 3 times higher in the 1 day extracts, regardless of whether comparisons were derived from the supernatant, membrane or the sum total of these extracts (Table 12 above).

The presence of endogenous reductants in samples could occasionally result in inhibition of haem staining, as described in section I. C) [3 ii d]. Comparing 1 and 3 day samples run on SDS-PAGE without (Figure 29a), and with (Figure 29b) prior solvent extraction, showed the inhibitory effect attributed to these endogenous reductants. Removal of these reductants by ethanol:acetone extraction was then required before cytochrome-c levels could be accurately quantified, and 1 day samples were then seen to contain higher levels of c-553 (Figure 29b). Quantification of c-553 levels on SDS-PAGE stained for haem peroxidase activity was essential as this was the only method that enabled the levels of membrane-associated as well as soluble c-553 to be assessed.

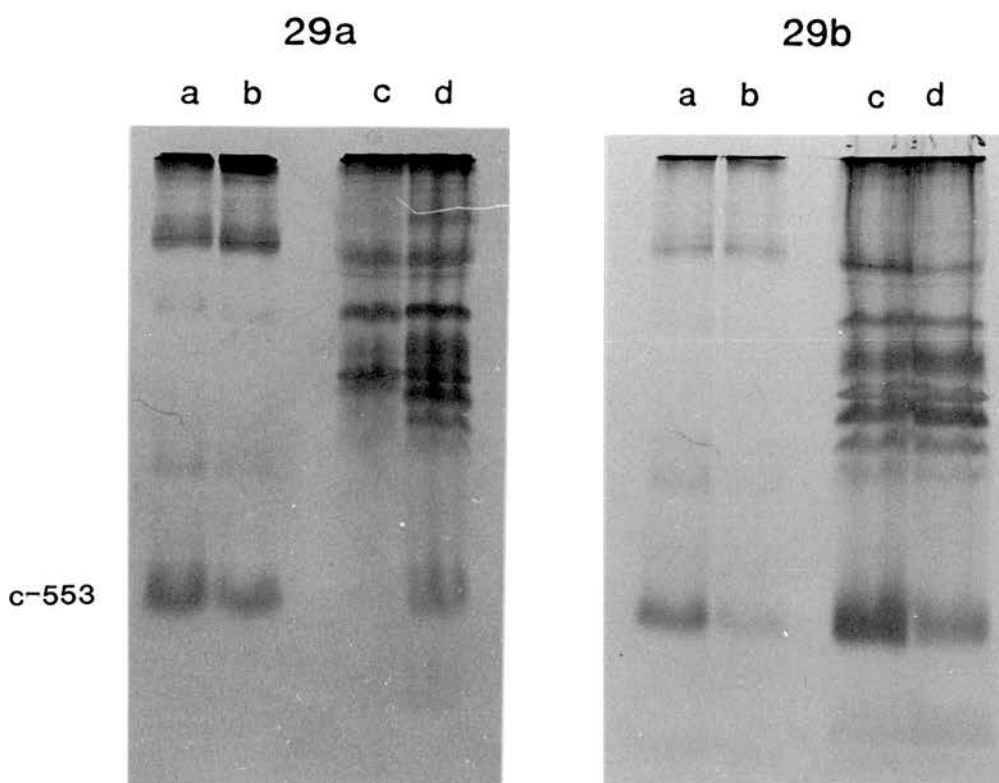


Figure 29. The effect on colour yields in haem-stained gels of ethanol-acetone extraction on *C.mucosalis* extracts. Electrophoresis was performed in gels containing 15% acrylamide and 0.4% bisacrylamide. Extracts from 1 and 3 day grown *C.mucosalis* were made by Freeze-thaw treatment. Samples applied to gel 29b were subjected to prior extraction with ethanol-acetone, samples in gel 29a were not so treated. Lanes a and b contain 1 and 3 day supernatants respectively. Lanes c and d contain 1 and 3 day membranes respectively. c-553 = Cytochrome c-553.

ii) Quantification of c-553 levels using Ascorbate-PMS Spectra.

When c-553 levels in cell supernatants were quantified by spectrophotometric analysis of the α -peak following treatment with ascorbate plus PMS, it was found to be preferable to carry out the reduction under argon. Atmospheric oxygen partially re-oxidised c-553, reduced with ascorbate-PMS, resulting in underestimates of the c-553 levels present.

Quantification, by reduction of the soluble fraction with ascorbate plus PMS (Figure 30), showed that extracts of 1 day cells had 1.4 times the c-553 present in extracts of 3 day cells.

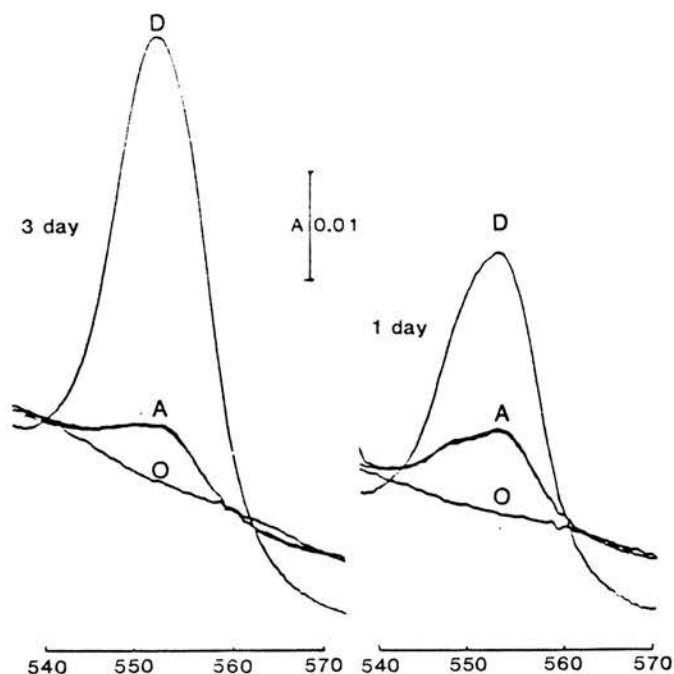


Figure 30. Quantification of cytochrome c-553 using ascorbate-PMS spectra. Absolute spectra of 1 and 3 day grown, freeze-thaw extracts were determined versus Na phosphate, pH7. The samples (in 80mM Na phosphate, pH7) were air oxidised (O), reduced with 1mM ascorbate/10mM PMS (A) and dithionite reduced (D). Cytochrome c-553 was quantified from ascorbate-PMS spectra using $E_{553}^{\text{red-ox}} = 15.59 \text{ mM}^{-1} \text{ cm}^{-1}$ and gave values for c-553 of 180 and 128 nmol.g cellular protein⁻¹ in 1 and 3 day extracts respectively.

iii) Quantification of c-553 isolated by Molecular Exclusion Chromatography.

Soluble fractions, prepared by freeze-thaw or French pressure cell treatment of C.mucosalis grown for 1 or 3 days, were applied to a G75 Sephadex column equilibrated and eluted with 10mM pH7 phosphate buffer. Fractions were monitored at 410nm to obtain the cytochrome-c profiles.

The A_{410} profiles derived from the French press extracts (Figure 20) contained considerable quantities of high M_r material, due to the formation of small membrane vesicles (Figure 3) which did not sediment on centrifugation and therefore remained suspended in the soluble fraction. The method used to release the soluble material did not however affect the quantification of c-553 as shown by the similar results obtained using freeze-thaw and French press treatments. The profiles of 1 and 3 day freeze-thaw extracts and those obtained by French pressure cell treatment are shown in Figure 20. Cytochrome c-553 was completely resolved by this column.

Samples of the fractions from the c-553 peak run on SDS-PAGE and stained for haem peroxidase activity revealed only one band, corresponding to the M_r of c-553, with no evidence for the presence of contaminating cytochromes-c. (An additional purification step of ion-exchange chromatography, confirmed that c-553 isolated on G75 Sephadex was not contaminated with other cytochromes.)

Spectral analysis of G75 fractions from the c-553 peak showed the asymmetric α -peak (Figure 31) characteristic of cytochrome c-553 (Goodhew et al. 1988). c-553 levels in the 1 and 3 day extracts, derived from the spectral analysis of pooled G75 fractions, are presented in table 13 below.

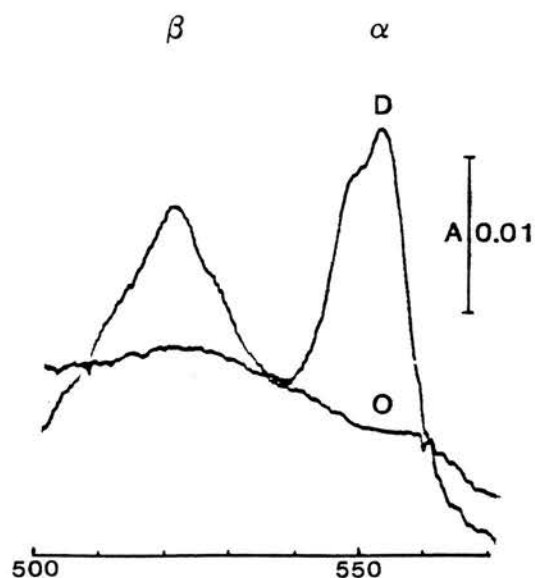


Figure 31. Spectral analysis of c-553 peak fractions isolated by G75 Sephadex chromatography. Air oxidised (O) and dithionite reduced (D) absolute spectra of fractions were made versus 0.1M Na phosphate, pH 7.5. The spectrum (which was typical of the spectra recorded) was that of fraction 75, derived from the chromatographic separation of a 1 day French-press extract shown in figure 20.

Table 13

Cytochrome c-553 quantification by spectral analysis of pooled Sephadex fractions.

	<u>nmol c-553 (g cellular protein)⁻¹</u>	
	<u>1 day</u>	<u>3 day</u>
Freeze-thaw	226	79
French-press	336	104

c-553 levels were therefore 2.8 to 3.2 times higher in the 1 day cells than in the 3 day growth.

Summary.

All of the quantification methods used, that is haem peroxidase activity on SDS-PAGE, ascorbate reducibility and molecular exclusion chromatography, gave comparable and consistent results. From these results it was concluded that C.mucosalis^{cultures} grown for 1 day contained a higher level of soluble c-553 than that found in ^{cultures} grown for 3 days. Therefore as the oxygen concentration falls, or as the cells age, cytochrome c-553 expression by C.mucosalis was repressed.

B) Repression of c-553 in the presence of catalase.

Growth of C.mucosalis on blood agar plates, which contain erythrocyte catalase, was compared with growth on serum agar, which does not. C.mucosalis was grown for 1 day, on blood agar and on serum agar plates contained in the same jar and therefore under identical gaseous conditions throughout. The levels of soluble c-553 induced were determined after French pressure cell treatment and centrifugation.

C.mucosalis grown in the absence of catalase contained higher levels of c-553 as determined from anaerobic ascorbate plus PMS spectra and from SDS-PAGE of the crude extracts stained for haem peroxidase activity and scanned at 690nm (Figure 32). G75 Sephadex molecular exclusion chromatography of the soluble extract was then used to isolate the c-553, and the spectrophotometric determination of pooled fractions confirmed that c-553 was induced in cells grown in the absence of catalase. The levels of c-553 were approximately 1.5 times higher than those found in cells grown in the presence of catalase.

Conclusions

The levels of c-553 induced in C.mucosalis correlate with the pattern of cytochrome-c peroxidase induction (Chapter 3), consistent with the proposed role for c-553 as physiological e^- donor to this terminal enzyme. This does not, however, preclude the alternative role as e^- donor to the cytochrome-c oxidase, which was also shown to readily accept e^- from c-553 (Chapter 4).

The role of c-553 as e^- donor to cytochrome-c peroxidase and cytochrome-c oxidase during the microaerobic respiration of C.mucosalis will be discussed in chapter 7.

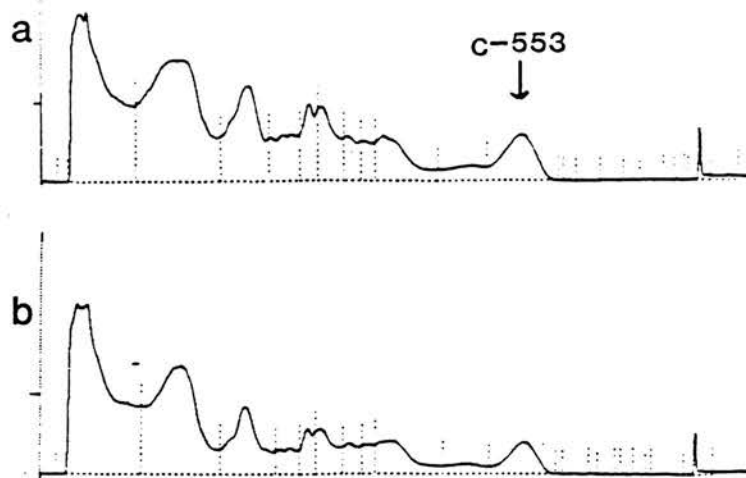


Figure 32. Repression of c-553 in the presence of catalase. *C.mucosalis* cells were grown for 1 day on agar plates supplemented with 5% horse blood (and therefore erythrocyte catalase) or 5% horse serum (thus without this enzyme). Soluble extracts were prepared by French-press treatment and samples separated in an SDS polyacrylamide gel containing 15% acrylamide and 0.4% bisacrylamide. The haem-stained gels were scanned at 690nm and the area of the c-553 peak (arrowed) determined from the scanner integration. The serum grown (a) and blood grown (b) extracts had c-553 peaks with areas of 18253 and 11200 respectively. a and b were loaded with samples (30ul) derived from broken cell suspensions containing 20mg protein per ml.

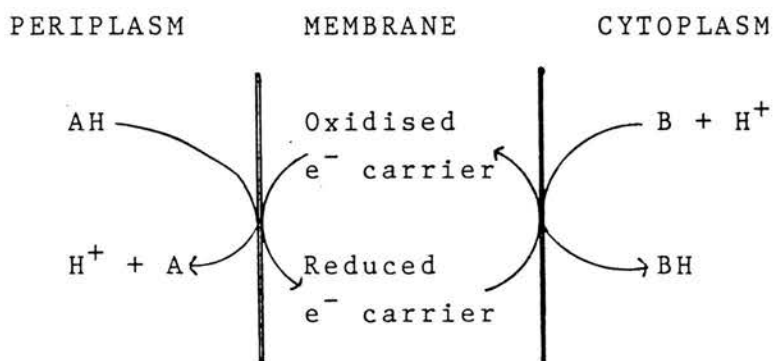
Chapter 6

Energetics

Introduction

Energy conservation by the production of ATP is a universal mechanism occurring in prokaryotes and eukaryotes. The formation of ATP can occur via substrate level phosphorylation and by respiratory chain linked oxidation of substrates whereby a proton gradient is developed and subsequently used for generating ATP. This chapter is concerned with the latter method of energy conservation.

The generation of a proton gradient across the cytoplasmic membrane of bacteria can be formed by e^- linked proton translocation from the cytoplasmic interior, to the outside, which is analogous to the H^+ transfer across the inner mitochondrial membrane. Proton gradients across bacterial membranes can also be generated by the sidedness of protons taken up and released during e^- transfer through the respiratory chain:-



Such reactions generate a net acidification of the external medium, which can be utilised to form ATP. Energy conservation in C.mucosalis by the formation of a proton gradient will be discussed in section IIIa.

The location of the respiratory enzyme's active site with respect to the cytoplasmic membrane can thus affect the potential generation of useful proton gradients. Knowledge of the location of proteins is thus of considerable interest for determining the action of respiratory chains. Cellular components can sometimes be localised by methods involving reaction product deposition or by the reaction of labelled antibodies raised against the protein of interest. Whole live cells can be used for the former methods if the substrate can gain access to its enzyme, or for the latter method if the antigen is located at the cell surface (Costerton 1979). As the outer membrane of gram-negative bacteria restricts the access of many molecules (DiRienzo et al. 1978, Caulcott et al. 1984), then this barrier would have to be removed before these techniques could be generally applied for the localisation of respiratory enzymes. Spheroplasts are formed by removing the cell wall and outer membrane in the presence of osmotic buffers so that the cytoplasmic membrane, containing the respiratory system, remains intact but exposed to experimentation. Intact and lysed spheroplasts can then be probed with respiratory substrates, to determine the topography of the constituent enzymes about the membrane. An additional benefit to be derived from the formation of spheroplasts is that membranes prepared by this technique retain higher respiratory activities compared to ultrasound or French press preparations (Probst 1980). Therefore experiments were carried out with the aim of forming spheroplasts from C.mucosalis. These experiments will be described in section I.

C.mucosalis (f. C.sputorum subsp. mucosalis) was originally isolated from porcine intestinal tissue (Lawson and Rowland 1974), and has been shown to invade and multiply within various tissue culture cell lines (Lawson et al. 1983). Most of the current experimental work on C.mucosalis was, however, carried out on bacteria grown on agar plates, an artificial growth medium. Although much valuable information regarding the respiratory system was obtained from using agar-grown C.mucosalis, it was desirable to investigate

bacterial respiration in situ within the host tissue, as the metabolism may differ from that seen within the isolated organism.

Tetrazolium salts, which are water soluble and near colourless in the oxidised form, will accept e^- from electron transport systems to form coloured, water-insoluble formazan compounds. If the oxidation of bacterial specific substrates could be linked to the reduction of tetrazolium salts then such a system would stain bacteria which were involved in active respiration, while leaving the mitochondria unstained. This differs from the fluorescent antibody technique generally employed to locate C.mucosalis (Rowland and Lawson 1974) which involves the reaction of antibodies raised against outer membrane proteins resulting in the staining of live and dead bacteria alike. Isolated agar-grown bacteria, uninfected and infected host tissues were investigated using various substrates and several tetrazolium salts, in the hope of developing a bacterial specific stain. These experiments will be described in section IIa.

Members of the genus Campylobacter utilise many organic and inorganic substrates and e^- acceptors, for example growth on H_2 or formate occurs, using O_2 , sulphur, nitrate, malate, thiosulphate or fumarate as e^- acceptors (Lawson et al. 1981, Laanbroek et al. 1978b, Niekus et al. 1978, Smibert 1978). C.sputorum subsp. bubulus, a species closely related to C.mucosalis, was found to produce H_2O_2 as well as H_2O from the oxidation of formate (Niekus et al. 1978, 1980a, 1980b, 1980c, Niekus and Stouthamer 1981). Experiments were performed with C.mucosalis showing that H_2O_2 was produced from the oxidation of formate, these experiments are described in section IIb.

The high level of H_2O_2 produced by C.sputorum subsp. bubulus at high dissolved oxygen tensions (d.o.t.) by a formate oxidase with low affinity for O_2 , was considered to be a major contributing factor to the microaerophilic nature of this organism (Niekus et al. 1980b). However, the aerotolerance of C.sputorum subsp. bubulus growing in the presence of formate did not differ from that seen when the

organism was grown with lactate in place of formate (Niekus et al. 1980b), even though production of H_2O_2 from lactate was less than 1% of that which occurred in the presence of formate (Niekus et al. 1980a). It was also noted that the addition of catalase to media containing formate, did not prevent the detrimental effects of a high d.o.t. on growth (Niekus et al. 1980b). However, the efficient scavenging of H_2O_2 by catalase was demonstrated, in my opinion, by the failure to obtain peroxidative oxidation of 2,2'-azino-bis 3-ethylbenzthiazoline sulfonate (ABTS) in the presence of catalase (Niekus et al. 1978).

It was suggested (Niekus et al. 1980b) that formate was tolerated by C.sputorum subsp. bubulus at favourable d.o.t., in spite of the inherent H_2O_2 production, although no active adaptive mechanism was proposed by which this phenomenon occurred, apart from the scavenging of H_2O_2 by the action of cytochrome-c peroxidase (Niekus et al. 1978, 1980b). In my opinion it seems possible that in the catalase-negative, microaerophilic Campylobacters, the production of H_2O_2 was tolerated due to the presence of an energy conserving mechanism involving e^- transfer to reduce H_2O_2 to H_2O . Experiments to determine if H_2O_2 , as well as O_2 , were capable of operating as terminal acceptors of e^- from the electron transport chain, for the generation of an energy conserving H^+ gradient will be described in section IIIa.

The presence of an energy conserving pathway involving H_2O_2 might explain both the absence of endogenous catalase, and the lack of protection conferred by exogenous catalase in catalase-negative Campylobacter species. The presence of catalase would remove H_2O_2 without the involvement of e^- transfer through the respiratory chain, hence depriving these organisms of energy conservation using H_2O_2 as terminal acceptor. The effect of exogenous catalase on C.mucosalis will be described in section IIIb, to determine if growth of the organism was adversely affected by the presence of this H_2O_2 scavenger in the growth medium.

SECTION I. TOPOGRAPHY OF RESPIRATORY CHAIN COMPONENTS.

Formation of bacterial spheroplasts.

A) Introduction. The structure of gram-negative bacteria.

Knowledge of the structure of bacteria is relevant to the successful production of spheroplasts. In outline the structure consists of a cytoplasmic membrane, containing the respiratory chain, which surrounds the cytoplasm. A cell wall, external to the cytoplasmic membrane, provides rigidity to the bacterium. External to the cell wall, in gram-negative but not in gram-positive organisms, is the outer membrane. Thus gram-negative bacteria are able to retain extracytoplasmic material between the outer and the cytoplasmic membranes, in an area which is generally referred to as the periplasmic space.

Formation of spheroplasts by the removal of the outer membrane while leaving the cytoplasmic membrane intact, releases into the medium enzymes and proteins that can be reliably identified as periplasmic. The intact spheroplasts can then be probed to reveal the presence of membrane-bound respiratory components that are periplasmic facing. Lysis of the spheroplast releases soluble cytoplasmic material and exposes cytoplasmic facing membrane components for experimentation.

This general definition of spheroplasts has not always been used. Other terms to describe such structures, which would now be referred to as spheroplasts, includes protoplasts and osmoplasts. Murray (1968) defined a protoplast as that in which the cell wall had been totally removed, whereas a spheroplast had its cell wall damaged in such a way as to become elastic and hence spherical. Osmoplasts (Asbell and Eagon 1966, Birdsell and Cota-Robles 1967, Voss 1964) were formed when bacteria retained their original shape, in a suitable buffer following cell wall treatment, but were however osmotically sensitive.

As will be seen, the removal or disruption of the outer membrane while still retaining an intact cytoplasmic membrane, is sufficient to release soluble periplasmic proteins into the external medium and to permit free access of substrates to the outer aspect of the cytoplasmic membrane. The presence or absence of the cell wall, and hence the shape of the structure, is largely irrelevant. The support provided by the cell wall can be replaced by an osmotically buffered medium, so as to prevent the turgor of the cytoplasmic contents (Costerton et al. 1974) from lysing the cytoplasmic membrane. Hence any structure that retains cytoplasmic contents, while releasing periplasmic proteins will be referred to in this thesis by the general term of spheroplast.

Some detail as to the biochemical composition of gram-negative bacteria is required to understand the methods used to form spheroplasts, and will be described below.

The cytoplasmic membrane consists of a phospholipid bilayer containing integral and peripheral proteins (Costerton 1979, Costerton et al. 1974). The membrane is a barrier for hydrophilic or charged molecules, unless an active transport system exists (Hancock 1984). When in a hypo-osmotic environment, the turgor pressure exerted by the cytoplasmic contents pushes the cytoplasmic membrane towards the supporting cell wall (Costerton et al. 1974, Costerton 1979).

The cell wall consists of peptidoglycan, which in gram-negative bacteria has an open molecular structure with few cross links (Costerton et al. 1974). The peptidoglycan is linked to the outer membrane non-covalently by proteins (Hancock 1984) and covalently via lipoproteins (Costerton et al. 1974, Hancock 1984). The peptidoglycan-lipoprotein complex and the periplasm therefore occupy the same region between the cytoplasmic and outer membranes. Early work showed a large portion of the periplasm to be in the form of a fluid filled space (Costerton et al. 1974). The peptidoglycan may however be much more hydrated than was thought previously, so forming a peptidoglycan gel that fills the periplasmic 'space' (Hobot et al. 1984). Proteins can diffuse freely through this gel,

but cannot move by convection (Hobot et al. 1984).

The outer membrane, like the cytoplasmic membrane, consists of a lipid bilayer studded with proteins (Costerton et al. 1974). Unlike the cytoplasmic membrane, the outer membrane contains lipopolysaccharide (LPS) (DiRienzo et al. 1978, Osborn and Munson 1974) as well as phospholipid. Recent work suggests that LPS is specific to the outer leaflet of the outer membrane bilayer, where it may, particularly in enteric bacteria, be the sole lipid component while, in non-enteric bacteria, LPS and phospholipid mixtures may occur (Nikaido and Vaara 1985, Hancock 1984). The absence of phospholipids in the outer leaflet of the membrane is thought to explain the resistance of enteric bacteria to phospholipases (Nikaido and Vaara 1985). The LPS carries a net negative charge (Hancock 1984) and is stabilised by divalent cations forming cross-links to other LPS molecules (Hancock 1984, Birdsell and Cota-Robles 1967, Costerton et al. 1974, DiRienzo et al. 1978, Nikaido and Vaara 1985).

In addition to the proteins and lipoproteins that anchor the outer membrane to the peptidoglycan layer, the outer membrane also contains porins which form water-filled pores (Hancock 1984, Caulcott et al. 1984, DiRienzo et al. 1978). Small hydrophilic compounds are taken up via the porins, with the size limit varying from organism to organism (Hancock 1984). Molecules with M_r up to around 650 gained access across the outer membrane of E.coli (Caulcott et al. 1984, DiRienzo et al. 1978). In P.aeruginosa, small molecules such as sucrose (M_r 342) may be partly restricted (Caulcott et al. 1984). This may be due to fewer numbers of pores, or to the presence of small pores (Hancock 1984).

As porins are water filled, the movement of small hydrophobic molecules through these pores is very restricted. A hydrophobic pathway through the outer membrane's lipid bilayer is very inefficient, which may be due to the divalent cation linked LPS leading to a high negative charge at the surface (Hancock 1984).

The properties of the cytoplasmic membrane, peptidoglycan

cell wall and the outer membrane must be taken into consideration to enable spheroplasts to be formed successfully. Removal of the cell wall should automatically be accompanied by disruption of the outer membrane, due to the structural links between these two structures. The breakdown of the cell wall will also release any periplasmic proteins that were present in the peptidoglycan matrix. Osmotic support must be provided prior to the cell wall treatment to prevent lysis of the cytoplasmic membrane following the removal of the supporting cell wall. Sucrose has generally been used (Bell et al. 1974, Birdsell and Cota-Robles 1967, Ingledew and Houston 1986, Mills and Bradbury 1984, Osborn et al. 1972, Osborn and Munson 1974, Neu and Heppel 1964a, Chatterjee and Williams 1963, Garrard 1971) and mannitol frequently used (Odom and Peck 1981a, 1981b, Kroger and Innerhofer 1976a, Jones et al. 1982) as osmotic supports. Both compounds are small (with M_r less than 350), and water soluble, and should therefore be able to cross the outer membrane via the porin channels into the periplasmic space. (However, see note on P.aeruginosa above.)

In addition to providing osmotic support, the presence of hypertonic solutions leads to plasmolysis of the cytoplasmic contents and hence shrinkage of the cytoplasmic membrane from the cell wall. Efficient plasmolysis was reported to be necessary for efficient spheroplast formation (Osborn et al. 1972, Osborn and Munson 1974, Birdsell and Cota-Robles 1967).

Spheroplasts (Kroger and Innerhofer 1976a) and cell lysates (Elliott and Greenwood 1984) have been produced by growing bacteria in the presence of β -lactam antibiotics, which prevent cell wall formation by inhibiting cross linking of the peptidoglycan (Stryer 1981). Removal of an existing cell wall, however, generally involves treatment with lysozyme which degrades the peptidoglycan (Murray 1968) by hydrolysis of the glycosidic link between the C-1 of N-acetyl muramic acid (NAM) and the C-4 of N-acetyl glucosamine (NAG) (Stryer 1981). Lysozyme has a M_r of approximately 14,000 and is therefore too large to traverse the intact outer membrane,

which must be disrupted before the enzyme can gain access to its peptidoglycan substrate.

Chelating agents are used to disrupt the outer membrane. These act by removing the divalent cations, in particular Mg^{++} , that stabilise the LPS-LPS interaction in the outer membrane (Birdsell and Cota-Robles 1967, Costerton et al. 1974, Hancock 1984, Nikaido and Vaara 1985). EDTA is the most effective (Neu and Heppel 1965) and widely used chelating agent, which is assisted by (Hancock 1984), or, at sufficiently high concentrations, can be partly replaced by TRIS (Neu and Heppel 1965). [Agents such as polymyxins and aminoglycosides that affect the permeability of the outer membrane, also act on the LPS-stabilising cations (Hancock 1984). As polymyxin ultimately destroys the cytoplasmic barrier (Nikaido and Vaara 1985) it is not a suitable agent for spheroplast formation.] Following EDTA treatment, the LPS has been reported to be largely released (Leive 1965), partly released (Hancock 1984), retained (Osborn and Munson 1974) or replaced by phospholipid molecules (Nikaido and Vaara 1985). Whatever the actual mechanism, EDTA was universally reported to disrupt the outer membrane thus permitting lysozyme to gain access to its substrate.

Treatment of gram-negative bacteria with sucrose, EDTA and lysozyme should result in the formation of spheroplasts.

B) Tests for spheroplast formation

i) Phase Contrast Microscopy.

The formation of spheroplasts has been monitored by viewing the morphology of the treated cells with phase contrast microscopes (Chatterjee and Williams 1963, Osborn et al. 1972, Birdsell and Cota-Robles 1967). This technique relies on viewing a representative sample and on counting a sufficiently large number of cells to enable valid conclusions to be drawn. As these preparations are unfixed (Birdsell and Cota-Robles 1967), then it is quite possible, in my opinion, that the cells will undergo changes during the accumulation of

data. In addition the shape of the bacterium may not change into a sphere during the formation of spheroplasts (Voss 1964). (see original definition of spheroplasts in section IA).

ii) Optical Density measurements to determine cell wall removal.

The optical density, measured at around 600nm, has frequently been used to estimate cell numbers in a bacterial culture or cell suspension. After removal of the cell wall, spheroplasts become sensitive to lysis in hypotonic media and the concurrent decrease in optical density can thus be used to estimate spheroplast formation. The stability of spheroplasts in osmotically buffered media is established by a relatively unchanging absorbance (Bell et al. 1974, Birdsell and Cota-Robles 1967, Fujita and Sato 1966a).

iii) Marker Enzymes.

Spheroplast formation is more usually (and in my opinion most reliably) monitored by assays to determine the release of marker enzymes. [Without such tests, it has been possible for erroneous claims to be made in the literature as to the location of certain proteins. For example Odom and Peck (1981b) reported that cytochrome-c₃ was periplasmic, cytoplasmic and membrane-bound in Desulfovibrio.]

Totally successful spheroplast formation would be indicated by release of all periplasmic contents with no release of cytoplasmic material into the medium. In practice the outer membrane and cell wall of some of the cells will resist the treatment, resulting in less than 100% release of periplasmic proteins and some contamination of periplasmic with cytoplasmic material will also occur due to some bacteria undergoing premature lysis of their cytoplasmic membrane. Figures of around 75-90% periplasmic release with 5-10% cytoplasmic contamination have been achieved with Thiobacillus (Ingledew and Houston 1986), Paracoccus (Alefounder and Ferguson 1981), E.coli (Neu and Heppel 1964b) Methylophilus

(Jones et al. 1982) and Spirillum (Garrard 1971).

Various enzymes and proteins were investigated to determine their suitability as markers for C.mucosalis and P.aeruginosa spheroplast experiments.

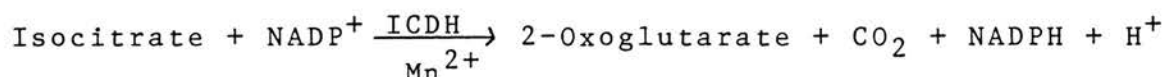
Cytoplasmic Markers

NAD and NADP linked enzyme reactions are probably cytoplasmic or cytoplasmic facing, as the co-enzymes would be liable to loss through the outer membrane if they were periplasmic (Hooper and Dispirito 1985). Most enzymes are specific, utilising either NAD or NADP, but not both.

Isocitrate dehydrogenase (ICDH) activity was used as a cytoplasmic marker in P.aeruginosa (Wood 1978) and Thiobacillus (Ingledew and Houston 1986). Malate dehydrogenase (MDH) was used in Paracoccus (Alefounder and Ferguson 1980, 1981) and in Spirillum (Garrard 1971).

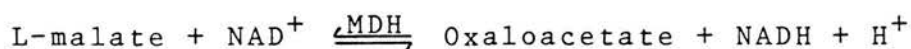
Both ICDH and MDH were assessed to determine if they were suitable soluble cytoplasmic markers in C.mucosalis and P.aeruginosa.

The ICDH assay (Materials and Methods) was based on that of Bergmeyer (1974). The reaction :-



was monitored by the increase in A_{340} resulting from the formation of NADPH.

The MDH assay (Materials and Methods) was that of Bergmeyer (1974).



At pH 7.5, the equilibrium of the reaction is far to the left (Bergmeyer 1974). The reaction, and hence the activity of MDH, was quantified by decreasing A_{340} as NADH was utilised in the presence of oxaloacetate.

After lysis of P.aeruginosa, ICDH activity was found principally in the soluble fraction, and was therefore

potentially suitable as a marker of the cytoplasm. The small residual activity (5% of the total) associated with membrane material was probably due to entrapment within the pellet. In P.aeruginosa, total ICDH activity was high at around 250mU (mg cellular protein)⁻¹. Contamination of a sample with even 5% of the cytoplasmic contents could easily be determined.

In C.mucosalis, ICDH activity was much lower than in P.aeruginosa. The pH of the buffer was varied to optimise the ICDH determination with the following results.

Table 14

C.mucosalis ICDH activity, Effect of pH

	pH 6.5	pH 7.5	pH 8.5
mU (mg cellular protein) ⁻¹	5.41	1.90	1.75

The activity of ICDH in C.mucosalis was therefore three times higher at pH 6.5 than at pH 7.5 or 8.5, but was still less than 5% of the activity in P.aeruginosa. The level of ICDH activity in C.mucosalis was therefore too low to enable it to be used easily as a cytoplasmic marker. There was also some evidence (table 15 below) that ICDH was partially membrane associated in this organism, which invalidates the use of the enzyme as a soluble marker.

Total MDH activity in C.mucosalis was approximately 50 fold higher than ICDH activity (table 15 below).

Table 15

MDH and ICDH activities in C.mucosalis and P.aeruginosa.

	mU (mg cellular protein) ⁻¹			
	<u>C.mucosalis</u>		<u>P.aeruginosa</u>	
	Total ^a	Soluble	Total ^b	Soluble ^c
MDH	670 (100%)	545 (81%)	228 (100%)	12 (5%)
ICDH	13 (100%)	7 (54%)	79 (100%)	106 (134%)

^a Total derived from the sum of membrane and soluble extracts prepared by lysozyme and EDTA treatment of C.mucosalis.

^b Total derived from the assay of a French press suspension of P.aeruginosa.

^c Soluble equals the sum of periplasmic and cytoplasmic fractions, following formation of EDTA/lysozyme spheroplasts.

[The enzyme activities of extracts prepared using the French pressure cell were frequently lower than in extracts prepared by lysozyme/EDTA treatment (as in the above table) or in extracts from C. mucosalis prepared by freeze-thaw treatment. This may be due to entrapment within vesicles (Figure 3) thus preventing the enzyme from gaining access to its substrate, or to denaturation of the enzyme by French press treatment itself. The latter phenomenon has been reported to occur (Probst 1980).]

MDH in C.mucosalis was at least 80% soluble and of high activity (table 15 above). This enzyme was used as a cytoplasmic marker for most of the spheroplast experiments on C.mucosalis.

In P.aeruginosa, MDH activity was as high or higher than ICDH activity, but was largely membrane-associated as only a small percentage was released into the soluble fractions (table 15 above). MDH was therefore unsuitable for use as a cytoplasmic marker in P.aeruginosa.

ICDH was therefore used exclusively as the cytoplasmic marker during spheroplast experiments with P.aeruginosa.

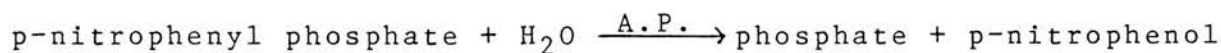
Periplasmic Markers

Soluble cytochromes-c (Wood 1983, Jones et al. 1982, Ingledew and Houston 1986, Fujita and Sato 1966a), ribonuclease (Neu and Heppel 1964b, Garrard 1971, Heppel 1967) and several phosphatases (Heppel 1967) are reported to be located in the periplasm, and are therefore candidates for use as marker proteins. Alkaline phosphatase was a commonly used periplasmic marker (Fujita and Sato 1966a, Malamy and Horecker 1961, Heppel 1967, Brockman and Heppel 1968, MacAlister et al. 1972, Neu and Heppel 1964b, 1965, Woolkalis and Baumann 1981, Schlesinger 1968, Garrard 1971). Alkaline phosphatase was generally released quantitatively into the medium on forming spheroplasts (Malamy and Horecker 1961, Garrard 1971). In Bacteroides ruminicola (Cheng and Costerton 1973), this phosphatase was found to be bound to a structural component of the extracellular matrix and hence not released on forming spheroplasts, but this phenomenon is unusual (see note section IA).

Probing with antibodies, raised against alkaline phosphatase, located the enzyme in the cytoplasm as well as the periplasm in E.coli (MacAlister et al. 1972). The cytoplasmic reaction was, however, due to enzymatically inactive subunits of alkaline phosphatase that dimerised to form the active enzyme after crossing the cytoplasmic membrane into the periplasm (Schlesinger 1968).

Alkaline phosphatase was the first choice to be investigated for use as a periplasmic marker in C.mucosalis and P.aeruginosa. The assay method was based on that of Bessey et al. (1946) using a Boehringer Mannheim colorimetric test combination kit and the '30 minute' method (Materials and Methods).

The reaction



was carried out under alkaline pH, the pH optimum for the

enzyme. The substrate is virtually colorless (Bessey et al. 1946), but on removal of the phosphate, p-nitrophenolate (A_{400} maximum under alkaline pH) is liberated. Determinations of serum alkaline phosphatase were carried out at a pH of approximately 10.5 (Boehringer, Bessey et al. 1946, Bergmeyer 1974). Determinations of alkaline phosphatase in E.coli were made at pH 8 (Malamy and Horecker 1961).

Extracts from C.mucosalis were assayed for alkaline phosphatase activity at pH 8, 9 and 10.5 to determine the optimum conditions (table 16 below).

Table 16

Alkaline phosphatase activity in C.mucosalis, effect of pH
mU (mg cellular protein)⁻¹

pH 8.0	0.48
pH 9.0	0.08
pH 10.5	0

The optimum pH was therefore pH 8 in C.mucosalis. The highest activity obtained was 0.8mU A.P.(mg cell protein)⁻¹ and 0.36mU A.P.(mg cell protein)⁻¹ for C.mucosalis and P.aeruginosa lysates respectively. The absorbance values for these assays were at best only 27% above the background (blank) values and would therefore be subject to high errors. Thus neither C.mucosalis or P.aeruginosa had sufficient alkaline phosphatase activity to enable this enzyme to be used as a periplasmic marker for spheroplast experiments.

The low activity of alkaline phosphatase in P.aeruginosa and possibly also in C.mucosalis, was probably due to repression of enzyme expression by the presence of inorganic phosphate in the growth medium. Repression of alkaline phosphatase was reported to occur in P.aeruginosa (Cheng et al. 1970), E.coli (Malamy and Horecker 1961) and in Vibrio species (Woolkalis and Baumann 1981). Phosphate starvation induced a 500 fold increase of alkaline phosphatase in P.aeruginosa (Cheng et al. 1970) and was required to visualise A.P. using ferritin linked antibodies in E.coli (MacAlister et

al. 1972). Alkaline phosphatase seems rarely to be constitutive, although this has been reported in Bacteroides (Cheng and Costerton 1973) and in a mutant of E.coli (Fujita and Sato 1966a).

In the current study, the growth medium used for P.aeruginosa contained 70mM inorganic phosphate which was therefore likely to repress the production of alkaline phosphatase. The agar media used to grow C.mucosalis contained no added phosphate and was processed, by Oxoid the manufacturers, to reduce the mineral content. The agar was supplemented with 5% horse serum or whole blood. Human serum contains 0.8-1.4mM phosphate, or more if haemolysis of the cells occurs (Gray and Howorth 1977). Supplemented agar plates probably contained at least 0.05mM phosphate, which may have been sufficient to repress alkaline phosphatase. C.mucosalis was found to grow poorly in the absence of blood or blood products, so it was not feasible to omit these from the growth media.

Alkaline phosphatase was therefore not suitable as a periplasmic marker for C.mucosalis or for P.aeruginosa grown in media supplemented with phosphate.

Soluble cytochromes-c were therefore used as periplasmic markers (see references above) for spheroplast experiments on C.mucosalis and P.aeruginosa. Spectral analysis of pure samples can be used to quantify c-type cytochromes. Quantification of crude extracts containing multiple cytochromes is less satisfactory, as extinction coefficients vary for different cytochromes-c. An 'average' value of $E^{550}_{\text{red-ox}} = 20\text{mM}^{-1} \text{ cm}^{-1}$ was used to estimate the cytochrome-c released on spheroplast formation.

C-type cytochromes retain their covalently bound haem in SDS and can be quantified by analysis of haem-stained SDS electrophoretic gels (see chapter 5 section I,C,3,ii, Goodhew et al. 1986). This technique was also used to assess spheroplast formation in C.mucosalis and P.aeruginosa.

C) Methods and Results of Spheroplast Experiments

i) Methods tested on P.aeruginosa

The method of Wood (1978) was used in this laboratory with minor modifications (the bacteria were washed after harvesting and the final bacterial cell density used was 100mg per ml), for the routine preparation of spheroplasts from P.stutzeri (Goodhew et al. 1986). This method (but using a cell density of 50mg per ml) could also be used to prepare spheroplasts from P.aeruginosa. Assays for ICDH showed that the periplasmic fraction (spheroplast supernatant) was contaminated with 5% of the cytoplasmic contents (table 17 below).

Table 17

Release of cytoplasmic marker enzymes from P.aeruginosa and C.mucosalis

	<u>P.aeruginosa</u>		<u>C.mucosalis</u>		M(+)	M(-)
	<u>ICDH (%)</u> S(+)	S(-)	<u>MDH (%)</u> S(+)	S(-)		
10mM Phosphate wash (pH7)	0	N.D.	3	N.D.	N.D.	N.D.
Periplasmic	5	0	61	50	5	2
Cytoplasmic	98	3	43	48	90	99
Membranes	6	12	N.D.	N.D.	N.D.	N.D.
French press extract	100	100	100	100	100	100

S = Sucrose as osmotic support.

M = Mannitol as osmotic support

(+) = Cells treated with Lysozyme and EDTA.

(-) = Cells treated without either lysozyme or EDTA.

N.D. = Not determined.

The haem-stained gel (Figure 33) shows the distribution of c-type cytochromes in these spheroplast fractions from P.aeruginosa. The soluble cytochromes c-551 and cd₁ were released following the conversion of the cells into spheroplasts and are clearly periplasmic. Low levels of soluble c-types also appear in the cytoplasmic fraction. This was likely to result from entrapment of periplasmic material

within the spheroplast pellet during centrifugation and probably also to bacteria that resisted the EDTA/lysozyme treatment, an aspect which will be discussed more fully below. The high level of c-type cytochromes and the low level of ICDH present in the periplasmic fraction indicate that a high proportion of the bacteria were converted into spheroplasts. This was confirmed by their osmotic sensitivity on transference from the sucrose buffered medium into TRIS/EDTA, which resulted in lysis of the cytoplasmic membrane as determined by the release of over 90% ICDH into the medium (table 17 above).

Lysozyme and EDTA were shown (Figure 33) to be necessary for the successful formation of spheroplasts from P.aeruginosa. Treatment without lysozyme or EDTA released little or no cytochrome-c into the sucrose buffered medium (Figure 33 channel 4), and the cells so treated were insensitive to osmotic shock releasing little cytochrome-c (Figure 33 channel 5) or ICDH into the 'cytoplasmic' fraction (table 17 above). The low activity of ICDH in the 'membrane' fraction also reflects the presence of large numbers of intact bacteria, as NADP (the co-enzyme for the ICDH assay) will be unable to cross the intact cytoplasmic membrane. [This phenomenon was shown to occur with NADP linked cytoplasmic enzymes in E.coli (Fujita and Sato 1966a)]

Formation of spheroplasts from P.aeruginosa was not, however, always achieved. Transferring cells from 0°C or 4°C to 30°C had to be avoided, as this thermal shock was found to cause premature lysis of the cytoplasmic membrane as shown by unacceptably high ICDH levels (24%) in the periplasmic fraction. However, the principal difficulty encountered with P.aeruginosa was resistance to formation of spheroplasts as shown by the low levels of soluble cytochrome-c released into the medium following lysozyme/EDTA treatment (Figure 34). Resistant bacteria frequently require a slight osmotic shock (Schwinghamer 1980, Osborn et al. 1972, Osborn and Munson 1974) to rupture the outer membrane and release the periplasmic contents. P.aeruginosa was subjected to such an osmotic shock

treatment, by dilution from 0.5M to 0.25M sucrose. This procedure aided the release of cytochrome-c from the periplasm by up to 3 fold, as determined by spectral analysis and from haem-stained gels (Figure 34). The additional cytochrome-c released was not associated with rupture of the cytoplasmic membrane, as ICDH in the periplasmic fraction was not increased and remained at under 1% of the total activity.

Conversion of P.aeruginosa into spheroplasts was therefore demonstrated to be possible, although care was required with the experimental conditions used to optimise spheroplast production.

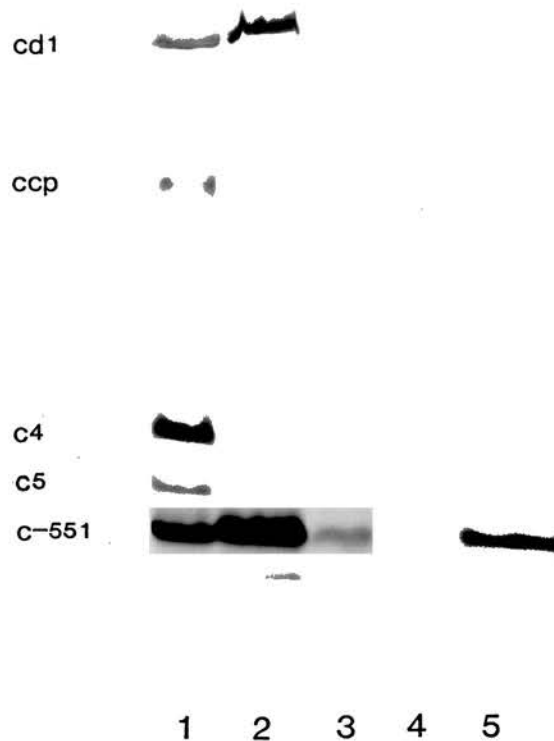


Figure 33. Assessment of spheroplast formation from Ps.aeruginosa using a haem-stained SDS polyacrylamide gel. The gel contained 15% acrylamide and 0.4% bisacrylamide. Lane 1 contains the soluble extract derived from French press treatment. Lanes 2 and 3 are the periplasmic and cytoplasmic fractions respectively, derived from treatment of a 50mg per ml cell suspension with EDTA and lysozyme by the method of Wood (1978). Lanes 4 and 5 are the 'periplasmic' and 'cytoplasmic' fractions respectively, derived from treatment of the cell suspension with neither EDTA nor lysozyme. Cytochromes cd₁, c₄, c₅, c-551 and cytochrome-c peroxidase (ccp) were located by reference to purified marker proteins (not shown).

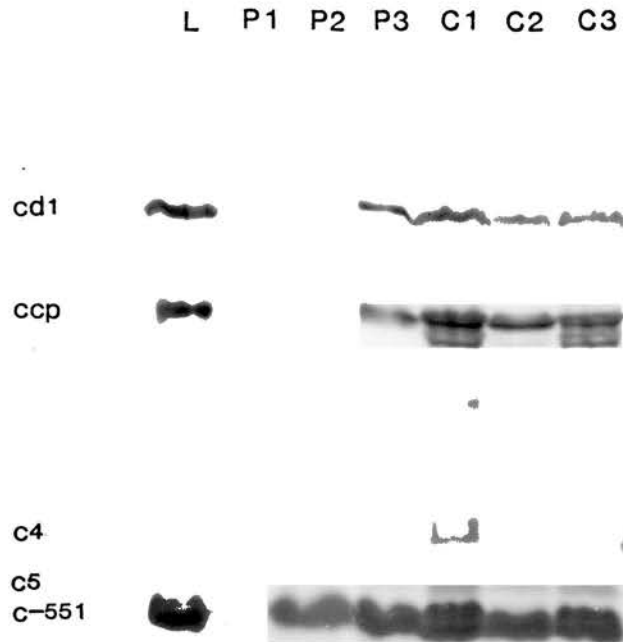


Figure 34. Resistance of *Ps.aeruginosa* to forming spheroplasts demonstrated using a haem-stained gel. The gel contained 15% acrylamide and 0.4% bisacrylamide. The samples were all adjusted to be equivalent to 30ul derived from a suspension of 100mg cells per ml. Lane L was treated with EDTA and lysozyme in the absence of the osmotic support (sucrose). Lane P1 was a periplasmic fraction derived by the method of Wood (1978) except that the cell suspension used was 100mg per ml. Lane P2 was a periplasmic fraction derived as P1 but with the addition of a mild osmotic shock from 0.5M to 0.25M sucrose, which was performed halfway through the 30 minute 30°C incubation period with lysozyme, EDTA and MgCl₂. Lane P3 was a periplasmic fraction derived as P2 but starting with a cell suspension of 200mg per ml. Lanes C1, C2 and C3 were cytoplasmic fractions derived from the spheroplast preparations P1, P2 and P3 respectively, by the method of Wood (1978). Cytochromes cd₁, c₄, c₅, c-551 and cytochrome-c peroxidase (ccp) were located by reference to purified marker proteins (not shown).

ii) Methods tested on C.mucosalis.

The method that was used successfully to form spheroplasts from P.aeruginosa (see above, section i) was tested without modification on C.mucosalis, that had been grown on Columbia Blood Agar plates. With C.mucosalis, however, a high level of the cytoplasmic marker MDH was released into the sucrose buffered medium ('periplasmic' fraction), indicating that the EDTA/lysozyme treatment had ruptured the cytoplasmic as well as the outer membrane (table 17 above). The cytoplasmic membrane of C.mucosalis was therefore prone to premature lysis during EDTA/lysozyme treatment even in the presence of sucrose as osmotic support. The method of Wood (1978) was therefore not suitable for the formation of spheroplasts from C.mucosalis.

Treatment of C.mucosalis with 1mM in place of 4mM EDTA still resulted in premature lysis of over 33% of the cells, and this occurred in the presence or absence of lysozyme. Of the remaining cells, which did not undergo premature lysis, around half (that is approximately 33% of the total) did not form spheroplasts as they failed to release their cytoplasmic contents following exposure to a hypotonic buffer. The maximum possible spheroplast formation was therefore only 33%, which was considered unacceptable. The results of this experiment indicated two conflicting problems, namely premature lysis of some bacteria while other cells were resisting the treatment altogether.

As noted above, experiments on P.aeruginosa indicated that the release of periplasmic proteins from resistant bacteria could be enhanced by the inclusion of a mild osmotic shock, without necessarily increasing premature lysis of the cytoplasmic membrane. The method of Osborn and Munson (1974) was chosen for use with C.mucosalis, as it combines an osmotic transition with a very slow addition of EDTA, which should have helped to minimise premature lysis of the cytoplasmic membrane. C.mucosalis cells, suspended in 0.75M sucrose/10mM TRIS pH 8 and treated with 100ug lysozyme per millilitre, were

diluted with 2 volumes of EDTA added by means of a peristaltic pump over several minutes. Final concentrations of sucrose and EDTA were 0.25M and 1mM respectively. In three separate experiments, MDH assays indicated that 60 to 80% of the cells underwent lysis of their cytoplasmic membrane within 5 minutes of the EDTA addition. This premature lysis was unaffected by the presence or absence of a washing step, or by the subsequent addition of lysozyme. These results suggested that C.mucosalis was particularly sensitive to EDTA.

A range of EDTA concentrations were then tried, to determine the optimum conditions that would result in the removal of the outer membrane and cell wall without concurrent lysis of the cytoplasmic membrane. The experiment was based on the assumption that spheroplasts, but not whole bacteria, would be osmotically sensitive thus releasing their cytoplasmic contents into the medium on suspension in hypotonic media. Whole bacteria and whole spheroplasts should retain their cytoplasmic contents when suspended in an osmotically buffered medium. Conditions that prematurely lysed the cytoplasmic membrane would, however, result in the release of cytoplasmic contents into osmotically buffered media.

C.mucosalis was suspended in 0.25M sucrose/3mM TRIS with 33mg cells and 33ug lysozyme per millilitre and EDTA present at 0 to 0.33mM. Premature lysis of the cytoplasmic membrane was determined by MDH assays of supernatants after centrifugation of osmotically supported samples. Spheroplast formation was determined by the increased release of MDH on suspending the cells in a hypotonic medium (10 fold dilution in distilled water). The results are shown in Figure 35a. The 'titration' curve for the supported cells shows that premature lysis increased rapidly as the concentration of EDTA was raised with virtually 100% lysis occurring in the presence of 0.33mM EDTA. The 'titration' curve for unsupported cells shows that at low concentrations of EDTA, many cells resisted the treatment and remained whole. The area between the two curves in Figure 35a represents the production of spheroplasts, which at best was only 25%, with the proportion of cells converted

into spheroplasts decreasing as the EDTA concentration was increased. The 'optimum' spheroplast formation occurred in the total absence of EDTA which still resulted in the premature lysis of 25% of the cells, with 50% of the cells remaining intact.

The experiment was repeated in the absence of lysozyme, but this only marginally increased the formation of spheroplasts. The 'optimum' EDTA concentration was 0.03mM (Figure 35b) which resulted in 32% spheroplast formation with 17% totally lysed cells and 51% whole bacteria. To ensure that premature lysis was not due to exposure of the microaerobic cells to atmospheric levels of O_2 , the experiment was repeated again without lysozyme, but maintaining the cells under a N_2 atmosphere during harvesting and EDTA treatment. This procedure did appear to decrease slightly the number of cells that lysed prematurely, but was associated with a increased number of cells that resisted the treatment completely. The result was that the proportion of spheroplasts produced was not increased (Figure 35c).

a

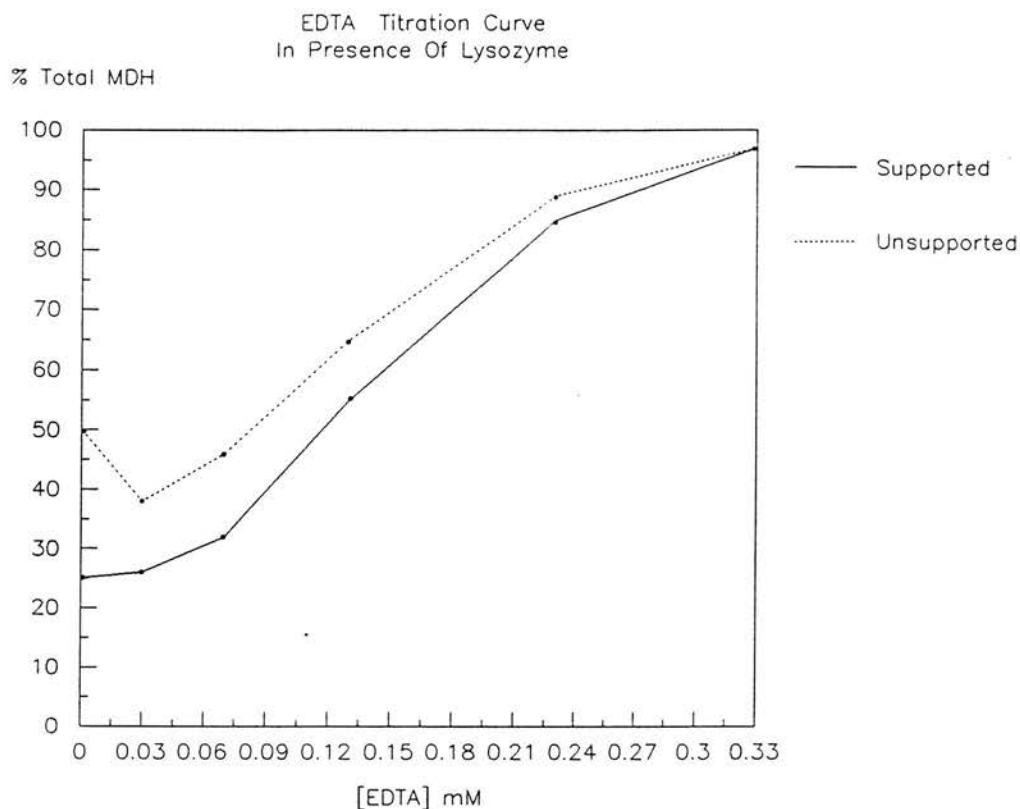
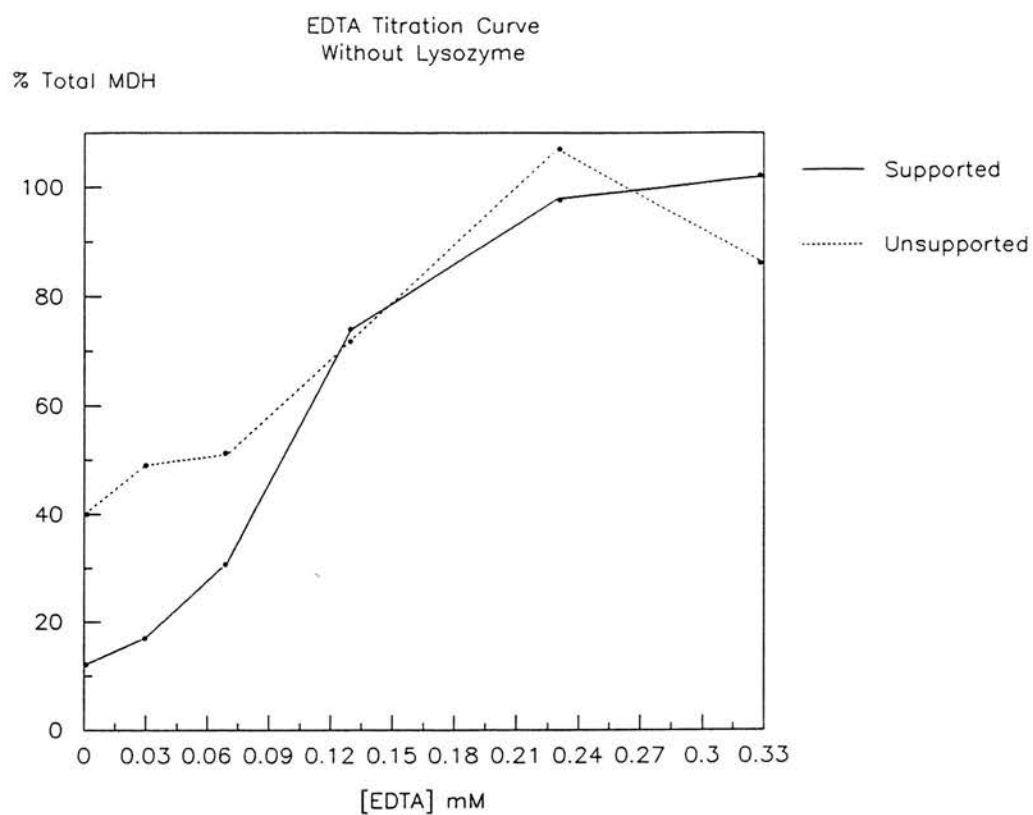
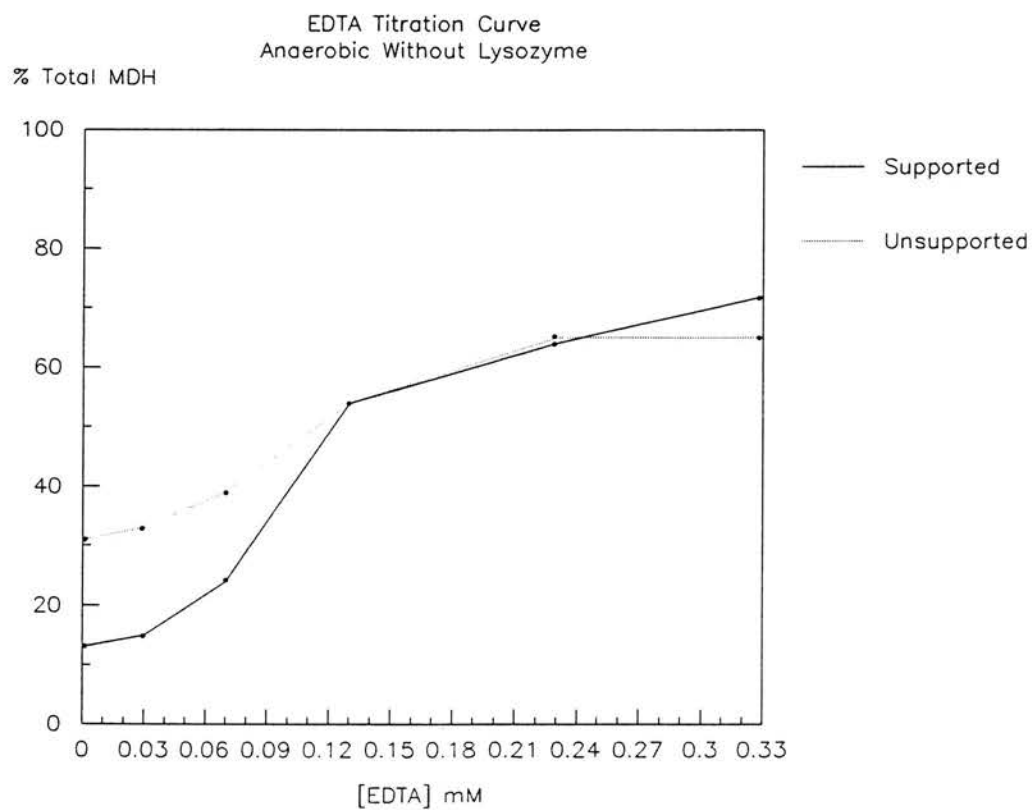


Figure 35. EDTA titration curves. 1 volume of a 100mg per ml *C.mucosalis* cell suspension supported in 0.25M sucrose, was treated with 2 volumes of EDTA in 0.25M sucrose/4.5mM TRIS-Cl (pH8) which was added slowly by means of a peristaltic pump. This treatment was a in the presence of lysozyme (33ug per ml), b and c in the absence of lysozyme and c under anaerobic conditions. The final EDTA concentrations were from 0 to 0.33mM, the final concentration of TRIS was 3mM. A sample of each treated cell suspension was diluted rapidly x 10 with distilled water to lyse spheroplasts so releasing the cytoplasmic marker MDH into the medium. The supported and unsupported (diluted) cell suspensions were centrifuged (30 minutes at 4000 x g) and the supernatants assayed for MDH which was calculated as a % of the total activity. The total MDH activity (100%) for the supported and unsupported cells were derived from assays of a freeze-thaw supernatant and of a 10 x dilution of this supernatant respectively. The freeze-thaw supernatant was from 1 volume of cells (100mg per ml in 0.25M sucrose) diluted with 2 volumes of 10mM Na phosphate, pH7, and was frozen and thawed twice.

b**c**

As the presence of EDTA seemed to be the causative agent for premature lysis of the cytoplasmic membrane, the possibility that spheroplasts might be formed by the action of lysozyme alone was investigated.

Sensitivity to lysis in unsupported media, as determined by declining optical density (see b ii above), was used to indicate experimental conditions necessary for the formation of spheroplasts from C.mucosalis. C.mucosalis cells were supported and plasmolysed by harvesting and washing in 0.5M sucrose/10mM TRIS. On suspension in 10mM TRIS, the cells appeared to be stable as the A_{650} remained relatively constant, thus indicating absence of cell lysis. Addition of lysozyme alone did not appear to result in the removal of the cell wall, as again the A_{650} remained steady (Figure 36a). Adding EDTA (0.5mM), however, rapidly resulted in a fall in the optical density (Figure 36a). From these results it was concluded that spheroplast formation would not occur on treating C.mucosalis with lysozyme in the absence of EDTA. Treatment of C.mucosalis with EDTA plus TRIS did appear to disrupt the cell wall, and the presence of lysozyme accelerated the effect. Addition of excess $MgCl_2$ to EDTA/lysozyme treated cells halted the fall in A_{650} , which seemed to confirm that EDTA or TRIS were the principal agents responsible for the removal of the cell wall. When added alone, neither TRIS or EDTA lysed the cells. Pre-treatment with TRIS followed by EDTA was more effective at producing osmotically sensitive cells than when the reagents were added in reverse order. It was concluded that treatment with TRIS/EDTA in the presence of an osmotic support could result in the formation of spheroplasts from C.mucosalis. [Prolonged exposure to TRIS should, however, be avoided as this was reported (Osborn and Munson 1974) to result in cell lysis. This effect was seen with C.mucosalis as one wash with 20mM TRIS resulted in approximately 0.5% MDH release, while a second and third wash each gave 5 times this level of cell lysis.]

EDTA/TRIS treatment, however, in the presence of 0.3M or 0.5M sucrose resulted in cell lysis which appeared to occur more rapidly than when the cells were osmotically unsupported (Figure 36b). That neither 0.2, 0.3 or 0.5M sucrose provided an effective osmotic support for the spheroplasts was confirmed by the high levels of MDH released (63%, 86% and 90% respectively) on treating the cells with EDTA or EDTA/lysozyme.

Treatment of cells with 0.5M sucrose/40mM TRIS/10mM MgCl₂ in the absence of EDTA or lysozyme also showed the extreme susceptibility of C.mucosalis to undergo lysis of its cytoplasmic membrane in the presence of sucrose as osmotic support. This treatment also made the remaining cells osmotically sensitive, as subsequent treatment of the pellet with hypotonic media (10mM TRIS/2mM EDTA/4mM MgCl₂) ruptured the cytoplasmic membranes of these cells. These results (Table 17) should be compared with the results obtained from P.aeruginosa when treated similarly. As sucrose did not appear to be effective with C.mucosalis, alternative osmotic supports were therefore tried for use with this bacterium.

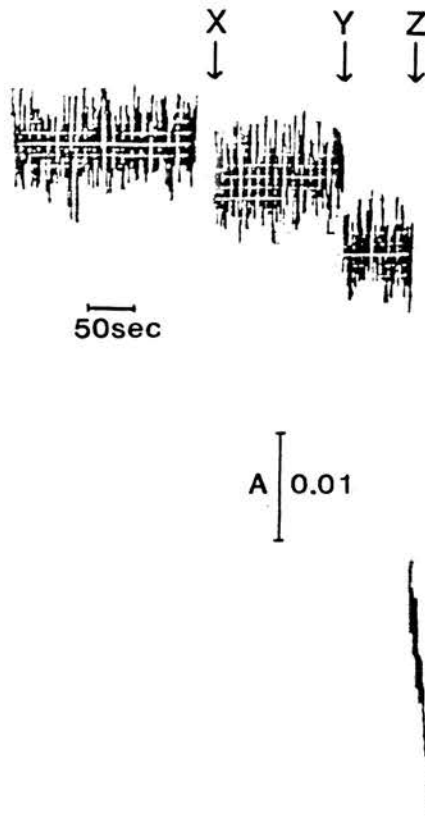


Figure 36a. Sensitivity of C.mucosalis to lysis in unsupported media, as determined by declining optical density. Cells were suspended at 100mg/ml in 10mM TRIS-Cl and placed in the cell and reference cell of the spectrophotometer. The optical density was monitored at 650nm. Lysozyme (to a final concentration of 200ug/ml) was added to the cell at X. The scan was halted at Y for 5 minutes and then resumed. EDTA (to a final concentration of 0.5mM) was added at Z which resulted in a rapid fall in the optical density indicating the occurrence of lysis.

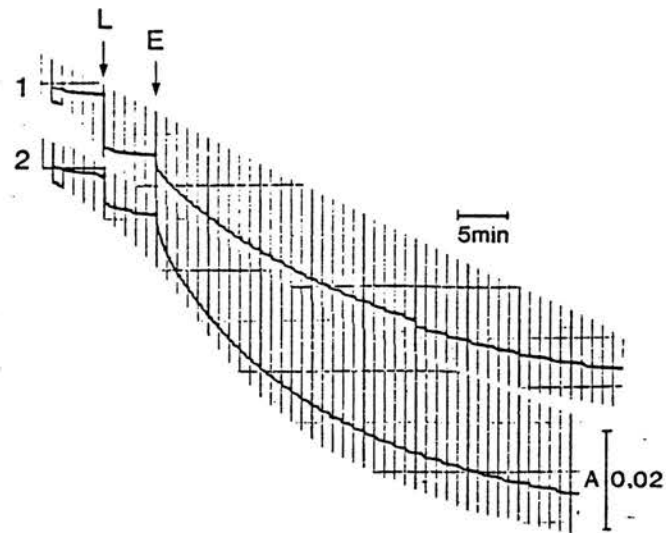


Figure 36b. Sensitivity of *C.mucosalis* to lysis in supported and unsupported media. *C.mucosalis* cells were suspended at 50mg/ml in 1 10mM TRIS-Cl or 2 10mM TRIS-Cl/0.5M sucrose. At L, lysozyme was added to a final concentration of 100ug/ml. At E, EDTA was added to a final concentration of 0.5mM. The decline in optical density was monitored at 650nm.

Cells suspended in 40mM TRIS without any additional osmotic support, were treated at 30°C with 0.5mM EDTA for 5 or 30 minutes and with EDTA for 5 minutes plus lysozyme for 30 minutes. EDTA treatment was terminated by the addition of 2.5mM MgCl₂. Determinations of MDH and cytochrome-c released into the 'periplasmic' fraction were made after centrifugation. The 'spheroplast' pellets were lysed by freeze/thaw treatment followed by suspension in 10mM TRIS/2mM EDTA. Centrifugation then separated the cytoplasmic from the membranous material which also contained any whole cells that resisted the treatment. The results are in table 18 below.

Table 18

Release of periplasmic and cytoplasmic markers from
C.mucosalis

	Activities as % Total					
	A		B		C	
	EDTA 5 min		EDTA 5 min +Lys		EDTA 30 min	
	MDH	Cyt.	MDH	Cyt.	MDH	Cyt.
Periplasmic	13	11	17	10	34	18
Cytoplasmic	63	28	64	32	39	20
Membranes	19	59	14	N.D.	22	58

Premature lysis of the cytoplasmic membrane, as determined by MDH in the periplasmic fraction (spheroplast supernatant), was much lower than in previous experiments when sucrose was present. Prolonged exposure to EDTA alone did result in lysis, with over 30% leakage of MDH when treatment was for 30 minutes (treatment C in table 18 above). Exposure to EDTA for 5 minutes (treatments A and B) released less than 20% MDH and the presence of lysozyme (B) had only a marginal effect on premature lysis.

Following treatments B and C the periplasmic soluble cytochrome-c was, however, released virtually in proportion to MDH activity, indicating that lysis of the outer and cytoplasmic membranes occurred together. Treatment with EDTA

alone for 5 minutes (A) did release a greater proportion of soluble cytochrome-c than MDH into the spheroplast supernatant, therefore some cells selectively underwent rupture of the outer membrane. However, the high level of cytochrome-c in the 'cytoplasmic' fraction shows that spheroplast formation was very incomplete. This may have been due to the absence of cell plasmolysis, which was reported to be necessary (Osborn and Munson 1974, Birdsell and Cota-Robles 1967) for the efficient formation of spheroplasts.

Mannitol, a hexose sugar of M_r 182, has been used as an osmotic support during the formation of spheroplasts (Kroger and Innerhofer 1976a, Odom and Peck 1981a, 1981b). Preliminary experiments, carried out in the absence of EDTA, showed that suspension of C.mucosalis and incubation for 30 minutes at 30°C in 0.5M mannitol in place of sucrose, resulted in a 75% reduction in the level of lysis.

For the preparation of spheroplasts, unwashed C.mucosalis were harvested and suspended at approximately 50mg cells per millilitre in 0.5M mannitol plus 10mM pH7 phosphate, and incubated at 30°C. A sample of this suspension was treated with 4mM EDTA/250ug lysozyme per ml/10mM $MgCl_2$ according to the protocol of Wood (1978), except that TRIS was omitted. Samples of the supernatants ('periplasmic') were taken by centrifugation of the suspension immediately after the addition of $MgCl_2$ (approximately 2 minutes incubation) and after 30 minutes incubation. The pellets were lysed to release the cytoplasmic contents by the method of Wood (1978), but omitting DNA'ase, then centrifuged to remove the membrane material. The release of MDH into the 'periplasmic' and 'cytoplasmic' fractions (as a percentage of the MDH in a French press supernatant) are in Table 17 above. The tabulated results are for the 30 minute incubation only. Incubation for 2 minutes gave very similar results.

Premature lysis of the cytoplasmic membrane was therefore low, as 5% or less of the MDH activity was present in the 'periplasmic' samples. 90 to 100% of the cytoplasmic contents were released following osmotic shock treatment with hypotonic

media. This indicates that there was a high level of susceptibility to osmotic shock, as would be expected following the formation of spheroplasts. Susceptibility to osmotic shock was not, however, dependent upon the presence of EDTA or lysozyme, as this occurred in cells that were treated solely with mannitol and phosphate. Thus C.mucosalis cells behaved quite differently from P.aeruginosa.

The release of periplasmic proteins from C.mucosalis was determined from the cytochrome-c profile of a haem-stained electrophoretic gel (Figure 37a). This shows that most of the cells did not release their soluble periplasmic cytochrome-c until subjected to the osmotic shock treatment which therefore lysed the outer and cytoplasmic membranes concurrently. The gel (Figure 37a) and MDH assay (Table 17 above) does indicate that a few spheroplasts were formed when the cells were treated for 30 minutes with lysozyme and EDTA, as some cytochrome-c was released without simultaneous MDH leakage. However, compared to P.aeruginosa (Figure 33) and P.stutzeri (Goodhew et al. 1986) spheroplast formation in C.mucosalis was very poor.

In an attempt to enhance the removal of the outer membrane and hence the release of periplasmic proteins, the experiment was repeated with some modifications. Concentrations of mannitol, lysozyme, EDTA and phosphate were as before. Treatment was in the presence or absence of 40mM TRIS and plus or minus a subsequent dilution with 1 volume of 40mM TRIS. Lysis of the 'spheroplast' pellets to release cytoplasmic contents was as Wood (1978). The results for the MDH released are in Table 19 below and are expressed as a percentage of a French-press supernatant.

Table 19

Release of the cytoplasmic marker MDH from C.mucosalis

	MDH release (%)			
	Minus dilution (+)TRIS	(-)TRIS	Plus dilution (+)TRIS	(-)TRIS
Periplasmic	14%	9%	15%	9%
Cytoplasmic	134%	125%	134%	124%

The table above shows that the leakage of MDH into the 'periplasmic' fraction was marginally increased by the presence of TRIS, which also resulted in slightly higher release of cytochrome-c (Figure 37b). The addition of the dilution step did not increase premature lysis, as MDH levels in the 'periplasmic' fraction were virtually identical in the presence or absence of this procedure (Table 19). The dilution step did not, however, appear to enhance the release of cytochrome-c either (Figure 37b).

From the pattern of cytochrome c-553 release it would appear from the gel (Figure 37b) that there was significant spheroplast formation, as all the 'periplasmic' fractions had higher levels of staining than was present in the 'cytoplasmic' fractions. For the high M_r cytochromes-c, however, this situation was reversed with their release occurring principally after treatment which lysed the cytoplasmic membrane. As all soluble c-type cytochromes studied are periplasmic (Wood 1983), then these results were anomalous. Possible explanations for these results include inhibition of staining and equilibrium of cytochromes-c between soluble and membrane phases.

The low level of c-553 that appears in the 'cytoplasmic' fraction (Figure 37b) may be due to the susceptibility of this cytochrome to reduction (and hence loss of iron from the haem in the presence of SDS) by endogenous reducing agents, as described in chapter 5. This effect would result in underestimation of cells that resisted the spheroplast treatment but not the lysis, which might more accurately be

reflected by the level of staining shown by the high M_r c-types. It is possible however that the high M_r soluble cytochromes sedimented with the spheroplast pellet and were therefore incorporated with the cytoplasmic fraction following lysis of the spheroplasts. On cell lysis, soluble c-type cytochromes should be released into the medium.

Membrane-washing experiments (Figure 19) have shown, however, that a proportion of high M_r cytochrome-c remains associated with the membrane, and can be gradually removed with repeated washing. Thus the high M_r cytochromes-c may be membrane-associated proteins that dissociate in the presence of lower ionic strength buffers such as those employed during treatment to lyse the cytoplasmic membrane.

P1 P2 P3 P4 C1 C2 C3 C4

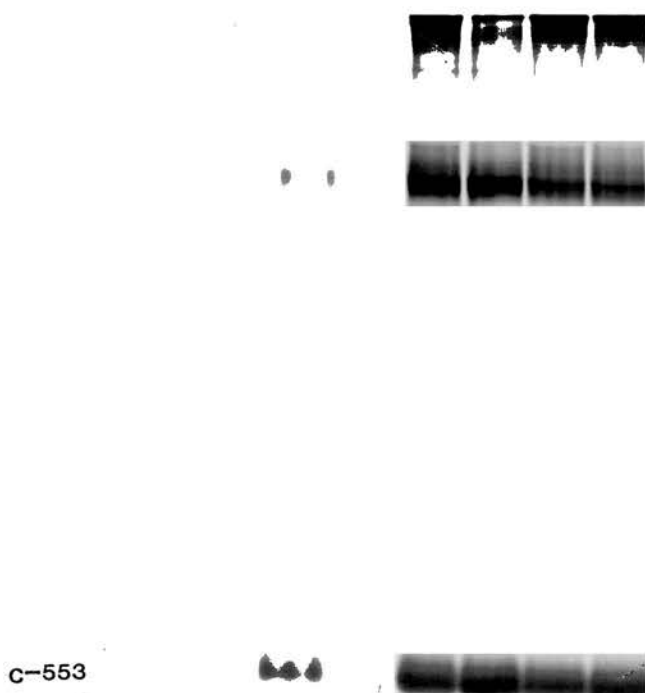


Figure 37a. Assessment of spheroplast formation from *C. mucosalis*. Gels (15% acrylamide/0.4% bisacrylamide) were stained for haem peroxidase activity. Cells were suspended at 50mg per ml in 0.5M mannitol. Lane P1 and P3 were periplasmic fractions derived from treating the cell suspension with 4mM EDTA and 250ug/ml lysozyme, with $MgCl_2$ added to 10mM after incubation at room temperature for 2 minutes. Lanes P2 and P4 were periplasmic fractions derived from treatment of the cell suspension without EDTA, lysozyme or $MgCl_2$. All treatments included incubation for 2 minutes at room temperature, lanes P3 and P4 were then treated at 30°C for 30 minutes. Lanes C1 to C4 were cytoplasmic fractions derived after the spheroplast treatments P1 to P4 respectively, by the method of Wood (1978).

All lanes were loaded with samples (30ul) derived from a cell suspension containing 4.3mg protein per ml.

c-553 = Cytochrome c-553.

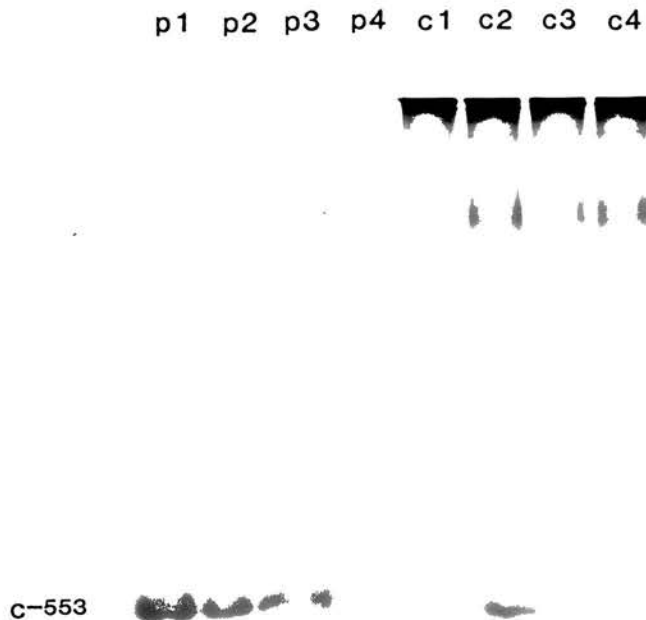


Figure 37b. Assessment of spheroplast formation from C.mucosalis. The gel and the treatment of the cell suspension was as described for sample P1 in figure 37a with the following exceptions. Incubation was for 45 minutes at room temperature. Lanes p1 and p3 contained 40mM TRIS-Cl (pH7.3) throughout. Cell suspensions used to derive the periplasmic fractions in lanes p3 and p4, were diluted with an equal volume of 40mM TRIS just prior to the centrifugation step to sediment the spheroplasts. The volume of sample loaded onto the gel was adjusted to compensate for the dilution step. Lanes c1 to c4 were cytoplasmic fractions derived from treatments p1 to p4 by the method of Wood (1978). All lanes were loaded with samples equivalent to 30ul derived from a cell suspension containing 4.5mg protein per ml. c-553 = Cytochrome c-553.

The absence of a reliable, specific periplasmic marker in C.mucosalis (compared to several soluble periplasmic c-types in P.aeruginosa see Figure 33), has made the interpretation of spheroplast experiments in this bacterium rather inconclusive. There is no doubt that C.mucosalis was much more prone than P.aeruginosa to spontaneous lysis during spheroplast treatments. 'Standard' procedures for the formation of spheroplasts that utilise sucrose and high concentrations of EDTA resulted in unacceptably high levels of MDH release, indicating that lysis of the cytoplasmic membrane had occurred. Using mannitol as the osmotic support may enable spheroplasts to be formed, but the efficiency cannot be easily quantified due to the lack of a specific periplasmic marker protein. It may be possible to reveal a specific periplasmic marker protein by means of protein staining, but this was not investigated for this project.

In theory it should be possible to form spheroplasts from all gram-negative bacteria. In practice some organisms, such as Methylophilus methylotrophus (Kasprzak and Steenkamp 1983) or organisms at a particular stage of growth (Neu and Heppel 1964b) or from a particular medium (Alefounder and Ferguson 1981), do not form spheroplasts. C.mucosalis did not form spheroplasts in spite of numerous attempts reported here. Campylobacter jejuni (Mills and Bradbury 1984) was also prone to lysis, as a 'spheroplast' preparation to produce pure outer membrane material was in fact contaminated with up to 40% cytoplasmic membranes. Thus, in the genus Campylobacter, it may be particularly difficult to produce spheroplasts. The technique was not investigated further.

SECTION II. RESPIRATION

IIa Tissue culture Experiments

A pig kidney cell line (provided by Rowland Okereke, Veterinary Pathology, R.D.S.V.S.) was grown as a monolayer in tissue culture and infected with C.mucosalis as described in Materials and Methods. Immunofluorescent staining of infected monolayers was by the method described in Rowland and Lawson (1974) and Lawson and Rowland (1974), using antisera raised against C.mucosalis and conjugated with fluorescein isothiocyanate (FITC). Conjugated antisera was provided by R. Okereke.

Preliminary studies determined conditions suitable for the maintenance of viable tissue cells during staining with tetrazolium salts. These are described in Materials and Methods.

Three tetrazolium salts, nitroblue tetrazolium (NBT), iodonitrotetrazolium (INT) and triphenyl tetrazolium chloride (TTC), were tested for use with tissue culture cells. NBT was reported (Horobin 1982) to be more readily taken up by tissues than was INT. As the formazan product of NBT is also more hydrophobic than that of INT (Horobin 1982), then NBT should be more suitable as a tissue stain. INT was however also tested, as its use might enable bacteria to be viewed by electron microscopy as well as by light microscopy, due to the presence of electron-dense iodine. In practice, NBT was found to be the only tetrazolium salt that gave adequate staining of tissues suitable for visualisation under the light microscope. NBT was therefore used for most of the subsequent experiments. Due to technical problems with the light microscope, it was not possible to produce good photographic records of the results. Extraction of the reduced formazans into acetone/HCl and optical density measurements were found, however, to confirm the subjective observations as to the intensity of

staining achieved.

The staining method was evaluated using C.mucosalis suspensions, uninfected and infected tissue cells, to attempt to find conditions that would specifically stain the bacteria while leaving the tissue mitochondria unstained.

Three substrates, H_2 , formate and succinate were used. In the absence of bacteria or tissue cells, none of these substrates reduced tetrazolium salts. In the presence of a bacterial suspension of C.mucosalis, reduction of tetrazolium salts occurred with all three substrates.

With formate, staining of C.mucosalis was sometimes enhanced when carried out under argon. This could be due to the inactivation of formate dehydrogenase (FDH) by O_2 or by H_2O_2 generated by formate oxidase activity under conditions of high oxygen tension (see section IIb), or to e^- flowing to a terminal oxidase, in addition to NBT, in the presence of oxygen. Staining levels in C.mucosalis grown for three days were more adversely affected by aerobic conditions than were cells grown for only one day. This might be due to the higher peroxidase activity in the 1 day cells (see chapter 3) removing H_2O_2 thus preventing damage occurring to the FDH enzyme. Alternatively, the higher oxidase activity in 3 day cells (see chapter 4) may lead to enhanced competition with NBT for the available electrons.

Unfortunately, tissue^{cultured} cells that were not infected with C.mucosalis also underwent staining with tetrazolium salts in the presence of all three respiratory substrates. The level of staining was initially lower with H_2 and formate than with succinate. However, after incubation for approximately 30 minutes, the level of staining was similar with the substrates formate and succinate. The presence of the respiratory inhibitors antimycin A or rotenone (at final concentrations of 0.7mg per millilitre), did not inhibit the staining of uninfected tissue cells. Endogenous substrates may, in part, have been responsible as some staining of the tissue was found to occur in the absence of added substrates. Pre-incubation of the tissues with phosphate buffered saline (PBS) to remove

endogenous substrates prior to the addition of tetrazolium salts was attempted. This resulted, however, in tissue cell death as determined by high levels of staining with the vital stain trypan blue, which stains nuclear material after cell death. After prolonged incubation in PBS, the dead tissue cell monolayer then became detached from the supporting cover-slip. Removal of endogenous reducing agents by pre-incubation was therefore not feasible.

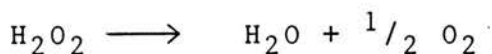
It was possible that C.mucosalis could be distinguished from tissue mitochondria by the different rates of staining that occurred when formate was used as substrate. With NBT and formate, C.mucosalis suspensions became heavily stained within a few seconds, while uninfected tissue remained only lightly stained even after 15 minutes incubation. Infected tissues, incubated with NBT and formate 29 hours post infection, did not, however, reveal the presence of any C.mucosalis bacteria either external or internal to the tissue cells, in spite of the presence of C.mucosalis in the infected tissues being confirmed by the fluorescent antibody staining technique. Pre-treatment with formalin permits binding of the antibody only to external C.mucosalis, while acetone treatment also allows access to internalised bacteria. Using these treatments it was shown that C.mucosalis cells were present, and that they were all internalised within the tissue culture cells. Unfortunately the fluorescence became quenched too rapidly to permit photography of these infected tissues.

In conclusion, the use of tetrazolium salts did not enable C.mucosalis to be specifically visualised when present within the host tissue. The absence of tetrazolium staining may have been due to the lack of viable bacteria, or to the absence of formate respiration in internalised bacteria. This method was not investigated further.

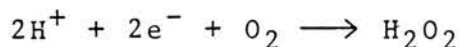
IIb Formate Oxidation

Formate oxidase activity was determined polarographically from the O_2 consumed by C.mucosalis cells and cell extracts in the presence of formate. With high levels of formate (10mM) the record of O_2 consumption was linear (zero order) between 254uM O_2 (in equilibrium with atmospheric O_2) and 10uM O_2 (Figure 38), which is indicative of an oxidase with a high affinity (low K_m) for oxygen. [In comparison, at O_2 tensions 25% of atmospheric, also in the presence of 10mM formate, the O_2 consumption by C.sputorum subsp. bubulus was reported to be around half of that observed in air-saturated buffer (Niekus et al. 1980b), thus indicating the presence of a low affinity oxidase in that species.]

The presence of H_2O_2 , that accumulated during the oxidation of formate by C.mucosalis, was demonstrated by O_2 released in the dismutation reaction catalysed by catalase:-



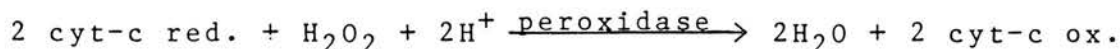
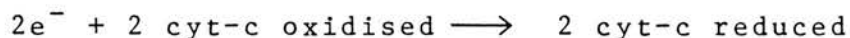
It should be noted that the dismutation reaction releases only half a molecule of O_2 for every molecule of H_2O_2 present, whereas the formation of H_2O_2 by the oxidation of formate consumes 1 molecule of O_2 :-



Therefore the quantity of O_2 released by the action of catalase represents only half of the O_2 consumed to form the accumulated H_2O_2 . The H_2O_2 formed, however, is not stable even in the absence of exogenous catalase. C.mucosalis does not contain catalase, but it does possess cytochrome-c peroxidase activity (Chapter 3), which will dispose of H_2O_2 . Hydrogen peroxide produced from the oxidation of formate will however

accumulate when the rate of H_2O_2 formation exceeds the rate of H_2O_2 removed by the action of cytochrome-c peroxidase.

The action of cytochrome-c peroxidase depends upon e^- , supplied from the electron transport chain, to reduce cytochrome-c. The oxidation of formate can also provide e^- for this reaction:-

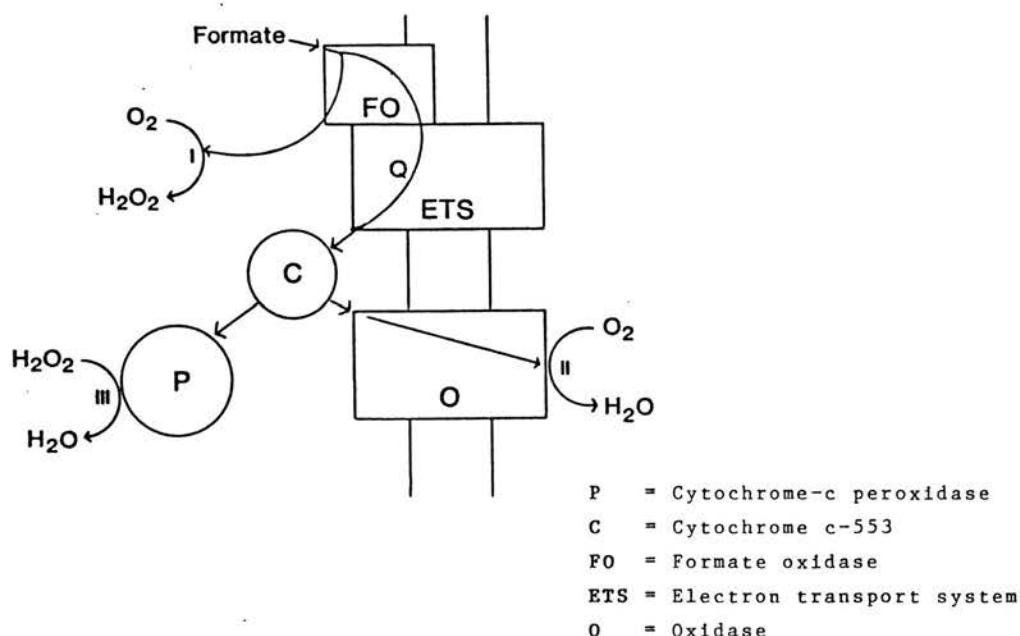


In the presence of excess formate, any H_2O_2 that accumulates during the oxidation of formate will, after the exhaustion of O_2 , become reduced to H_2O by the continuing action of cytochrome-c peroxidase under anaerobic conditions. Inhibiting e^- transfer to the cytochrome-c peroxidase should, in theory, result in the stable accumulation of all the H_2O_2 formed during the oxidation of formate. Enhanced accumulation of H_2O_2 was shown to occur when formate oxidation proceeded in the presence of the respiratory inhibitor, 2-heptyl 4-hydroxyquinoline N-oxide (HOQNO) (Figure 38). HOQNO was reported to inhibit e^- transfer between cytochrome b and a quinone (Kroger and Dadak 1969, Kroger and Innerhofer 1976a, 1976b) or at the level of the cytochrome b itself (Kroger et al. 1979), thus inhibiting cytochrome-c reduction. Although the inclusion of HOQNO enhanced the accumulation of H_2O_2 , presumably by restricting supplies of reduced cytochrome-c to the peroxidase, there was the added effect of inhibiting O_2 utilisation which dropped from 313 nmol O_2 mg protein $^{-1}$ in the absence of HOQNO, to 162 nmol O_2 mg protein $^{-1}$ in the presence of the inhibitor. [In the related C.sputorum subsp. bubulus when formate oxidation via the respiratory chain was inactivated using glutaraldehyde, formate oxidase activity was reported to remain relatively intact (Niekus and Stouthamer 1981). However, to obtain a comparable rate of oxygen

utilisation as occurred with untreated cells, these authors used 50% more of the glutaraldehyde treated cells, in terms of cellular protein. This suggests that oxidase activity was substantially affected by the treatment.]

The records of O_2 consumption by C.mucosalis in the presence of HOQNO were also non-linear (Figure 38), revealing a higher critical O_2 concentration, which is the point at which oxygen consumption ceases to exhibit zero-order kinetics (White 1963). These effects can be explained with reference to the diagram below giving the proposed routes of e^- transfer in C.mucosalis.

Proposed routes of electron transfer in C.mucosalis



The lower rate of O_2 utilisation and the accumulation of H_2O_2 were probably due to inhibition of the respiratory chain at Q, presumably at the level of quinone involvement. This would result from O_2 utilisation occurring only at I and not at II, with the H_2O_2 produced by reaction I accumulating due to blockage of e^- transfer to III. The non-linear record of O_2 consumption that was observed in the presence of HOQNO (Figure 38) can be explained by progressive inhibition occurring at Q.

An alternative explanation of the results is that I has a

low affinity for O_2 while II has a high affinity for O_2 . Thus inhibition by HOQNO at Q would lead to O_2 consumption solely via the low affinity oxidase (I). This would result in lower initial rates of O_2 utilisation, H_2O_2 accumulation and non-linear O_2 consumption at low oxygen tensions.

In conclusion, the effect of HOQNO was to inhibit oxygen utilisation, as well as the supply of e^- to the cytochrome-c peroxidase. Inhibition of the H_2O_2 -removing peroxidase system, while H_2O_2 production continued to occur, led to H_2O_2 accumulation. Production of H_2O_2 by C.mucosalis during the oxidation of formate was clearly demonstrated.

To avoid the problems associated with the use of HOQNO, an alternative method for eliminating cytochrome-c peroxidase activity was attempted. High levels of cytochrome-c peroxidase activity were found in soluble extracts of C.mucosalis (see chapter 3). Separation of the membrane fraction, which contains the formate oxidase activity, from the soluble fraction, which contains high peroxidase activity, should therefore result in enhanced H_2O_2 accumulation during the oxidation of formate. The results of these experiments are shown in Figure 38. There was some accumulation of H_2O_2 by the isolated membranes, but it was not as pronounced as that which occurred in whole cells inhibited with HOQNO. This result may reflect the presence of membrane-associated cytochrome-c peroxidase activity removing H_2O_2 . Unfortunately the cytochrome-c peroxidase activity of membrane extracts could not be determined, due to the technical problems described in chapter 3, and so the extent to which H_2O_2 was removed by the action of a membrane-bound peroxidase, remains uncertain.

Formate oxidase activity in the membrane extract was lower than that seen in the whole cell suspension (Figure 38). The soluble fraction itself was devoid of any formate oxidase activity, but the requirement for a soluble co-factor cannot be discounted.

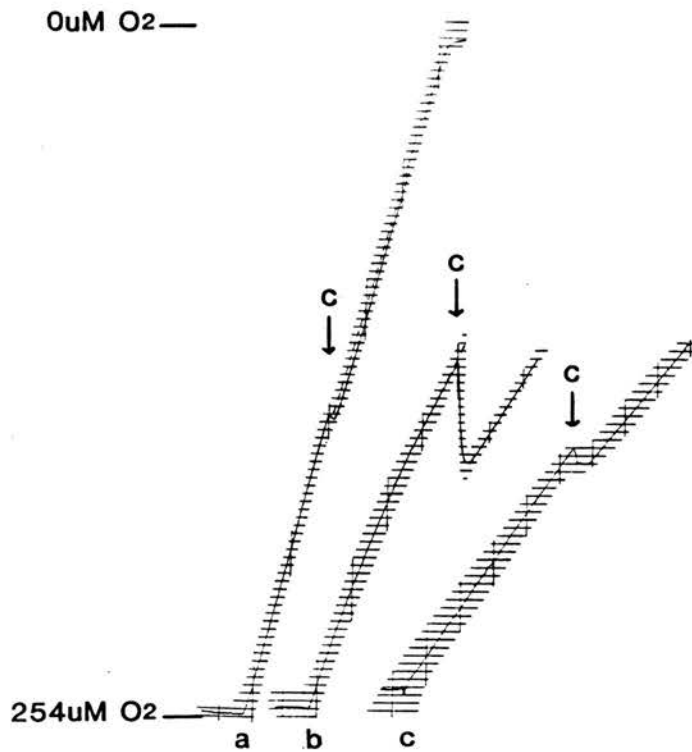


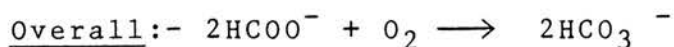
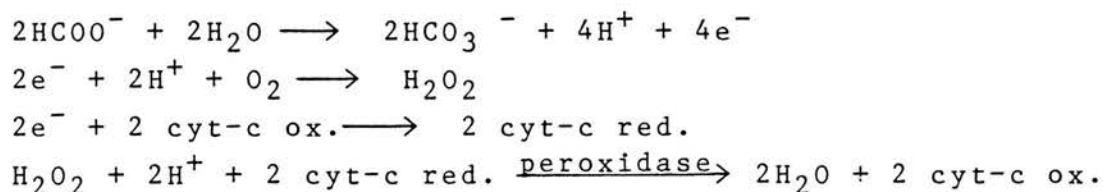
Figure 38. The oxidation of formate by *C.mucosalis*. Oxygen consumption by cells was determined at 25°C in the presence of 10mM Na formate/10mM Na phosphate/1mM EDTA (pH7) in a final volume of 2ml. The reaction was initiated by the addition of 50ul of a 1 day cell suspension (a and b) or membranes (c) derived from a broken cell suspension containing 20mg protein per ml. 1400 Sigma units of catalase were added at C. The record of O₂ consumption in b) was obtained in the presence of 50uM HOQNO.

The stoichiometry of formate oxidation

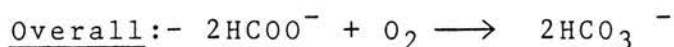
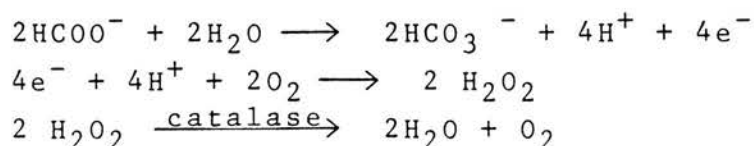
In the above assays, high levels of formate were used to demonstrate the capacity for H_2O_2 production by C.mucosalis. The stoichiometry of the reaction cannot, however, be elucidated from these traces. The method of Niekus and Stouthamer (1981) was used to determine the amount of O_2 consumed for each molecule of formate oxidised, and the proportion of H_2O_2 produced from such a reaction. A small (250nmol) 'pulse' of formate was added to C.mucosalis cells, and O_2 was consumed until the substrate was exhausted (Figure 39a). H_2O_2 that accumulated during the reaction was then estimated, as before, from the oxygen released after the addition of catalase.

The oxidation of formate can be described by 3 sets of equations. These are based on the reactions described by Jacobs and Wolin (1963b), Niekus et al. (1978, 1980b).

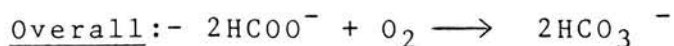
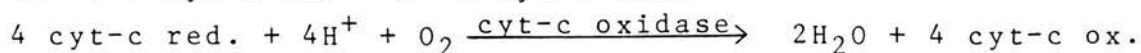
1) Formate oxidation to H_2O_2 with subsequent peroxidative reduction to form H_2O .



2) Formate oxidation to H_2O_2 , then dismutation by catalase to form H_2O and O_2 .



3) Oxidation of formate direct to H₂O.



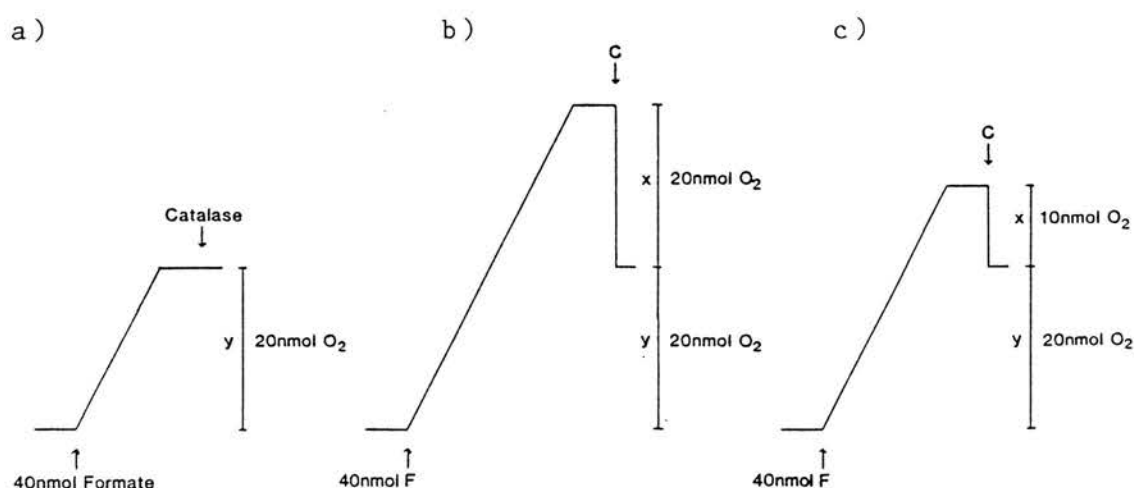
Thus after the addition of catalase, the ratio of mol formate oxidised per mol O₂ consumed should be 2, regardless of the route or combination of routes by which formate oxidation occurred. The measured stoichiometries (HCOO⁻/ O₂) in Figure 39 a,b,c, were 2.11, 2.22 and 2.06, which correspond quite well to the expected figure of 2.

The records of O₂ consumption, before and after H₂O₂ dismutation by catalase, can be used to calculate the proportion of formate oxidised to H₂O₂ and the proportion oxidised to H₂O. The calculation can be illustrated by theoretical traces where there was:-

a) 100% oxidation of formate to form H₂O.

b) 100% oxidation of formate to form H₂O₂

c) Partial oxidation to form H₂O, and partial oxidation to form H₂O₂



In a) the stoichiometry (HCOO⁻/ O₂) is 2, as the formate is

oxidised directly to H_2O . In b) before the addition of catalase, the ratio of $HCOO^- / O_2$ is 1 as all the formate is oxidised to form H_2O_2 , but after the addition of catalase (and the dismutation of H_2O_2 to H_2O) the ratio is 2. In c) the ratios are 1.33 and 2 before and after the addition of catalase respectively. Thus in c) the proportion of formate oxidised to H_2O_2 is not immediately apparent from the $HCOO^- / O_2$ ratio. However, the percentage of e^- flowing to form H_2O_2 equals $x/y \times 100$

a)	b)	c)
0/20 = 0%	20/20 = 100%	10/20 = 50%

This calculation will represent the true percentage flow of e^- from formate to form H_2O_2 only if peroxide accumulation is stable. In reality, the activity of cytochrome-c peroxidase will result in the removal of some or possibly all of the H_2O_2 formed, and hence the calculation underestimates H_2O_2 production. H_2O_2 accumulation should however reflect production when HOQNO is used to inhibit the supply of e^- to the cytochrome-c peroxidase.

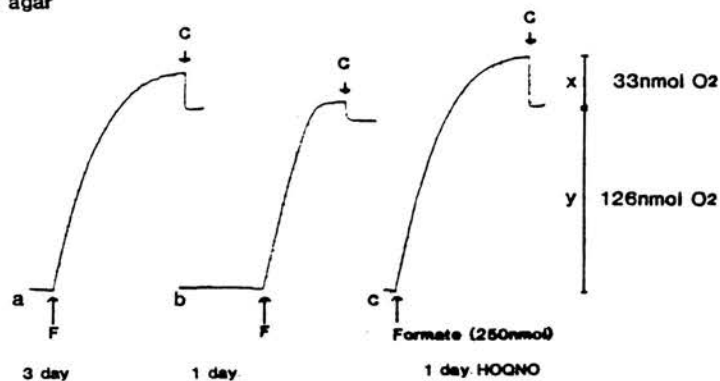
In the absence of inhibitors, C.mucosalis cells grown for 3 days accumulated more H_2O_2 than did cells harvested after only 1 days growth (Figure 39). In the presence of the respiratory inhibitor HOQNO, the accumulation of H_2O_2 by the younger cells was greatly enhanced whereas the effect on the older ^{cultures} was less pronounced. These results were consistent with the findings reported in chapter 3, namely that young cells have higher cytochrome-c peroxidase activity which, in the absence of inhibitors, allows removal of H_2O_2 at the same rate as it is formed. The high levels of H_2O_2 that accumulated with young cells incubated with HOQNO shows that they have the capacity to produce higher levels of H_2O_2 than do the older cells.

However a different pattern was obtained for serum plates (Figure 39) which indicated that the older cells had a higher capacity for H_2O_2 production. Such serum-grown cells were shown (Chapter 3) to contain very high levels of cytochrome-c

peroxidase activity. It was possible that partial HOQNO inhibition could lead to significant H_2O_2 removal, particularly with the young cells. Partial inhibition, by HOQNO, of respiratory chain activity was reported in Wolinella succinogenes (Jacobs and Wolin 1963a, Kroger and Innerhofer 1976a) and in E.coli and Pr.vulgaris (Lightbown and Jackson 1956).

Production of H_2O_2 by C.mucosalis during the oxidation of formate was shown conclusively, and the capacity of young cells to produce higher levels of H_2O_2 than older cell was demonstrated for cells grown on blood agar.

Blood agar



Serum agar

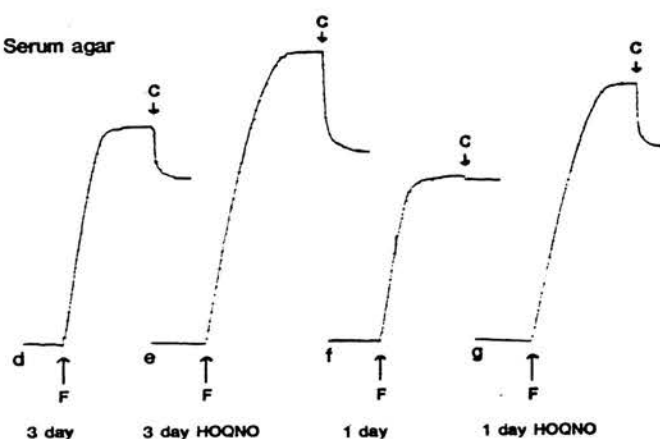


Figure 39. The stoichiometry of formate oxidation by *C. mucosalis*. Cells were grown on blood agar plates (a,b,c), or on serum plates (d,e,f,g). The chamber contained 50ul cell suspension (20mg protein per ml) in 10mM Na phosphate/1mM EDTA (pH7). Formate pulses (250nmol) were added to cells grown for 1 day (b,c,f,g) or for 3 days (a,d,e). HOQNO (50uM) was preincubated with the cells in c,e and g. After the substrate was exhausted, 1400 Sigma units of catalase were added (C).

SECTION III. ENERGY CONSERVATION.

IIIa Respiration-linked proton translocation in *C.mucosalis*

Respiration-linked proton pumping across the cytoplasmic membrane results in acidification of the external medium, which should be detected by sensitive pH electrodes. Leakage and ATP-synthase activity rapidly collapses a pH gradient therefore pH changes, due to substrates oxidised via the respiratory chain, will be transitory.

Proton translocation in *C.mucosalis* was investigated using a method based on that of Dawson and Jones (1981a). The chamber was stirred, maintained at 27°C and anaerobic conditions were achieved by flushing with oxygen-free nitrogen. Acidification of the lightly-buffered medium, 3mM N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), was recorded by a sensitive pH electrode connected to a chart recorder. The system was calibrated by additions of 0.01M HCl or 0.01M KOH, which gave a linear plot between pH 6.8 and 7.2 (Figures 40a and 40b). All experiments were performed within these pH limits.

The method was tested with freshly prepared rat liver mitochondria. The medium for experiments with mitochondria contained sucrose, to provide osmotic support, valinomycin plus KCl to dissipate the membrane potential component of the proton motive force, and N-ethylmaleimide (NEM) to block the OH⁻/phosphate antiporter (H⁺/phosphate symporter) (Brand et al. 1976). Succinate was present as the respiratory substrate, and rotenone was included to block the oxidation of endogenous substrates via NADH dehydrogenase.

The terminal e⁻ acceptor O₂ was added by injection of 150ul air-saturated buffer, which contained 39nmol O₂. This resulted in the acidification of the medium (Figure 41), which demonstrated proton translocation by mitochondria. The ability of the mitochondria to pump protons was, however, short lived presumably due to progressive membrane damage, uncoupling e⁻

transport from vectorial H^+ translocation. Membrane damage was probably also the cause of the low measured ratio of H^+ translocated per O atom added (H^+/O or $H^+/2e^-$). The maximum value of H^+/O recorded was 1.8, which gives a value of approximately one H^+ translocated per site. This value is much lower than the $2H^+$ translocated per site (Mitchell and Moyle 1967), which in itself is now thought to be an underestimate (Brand et al. 1976). The results obtained (Figure 41) showed, however, that the system was capable of qualitatively demonstrating H^+ extrusion by mitochondria.

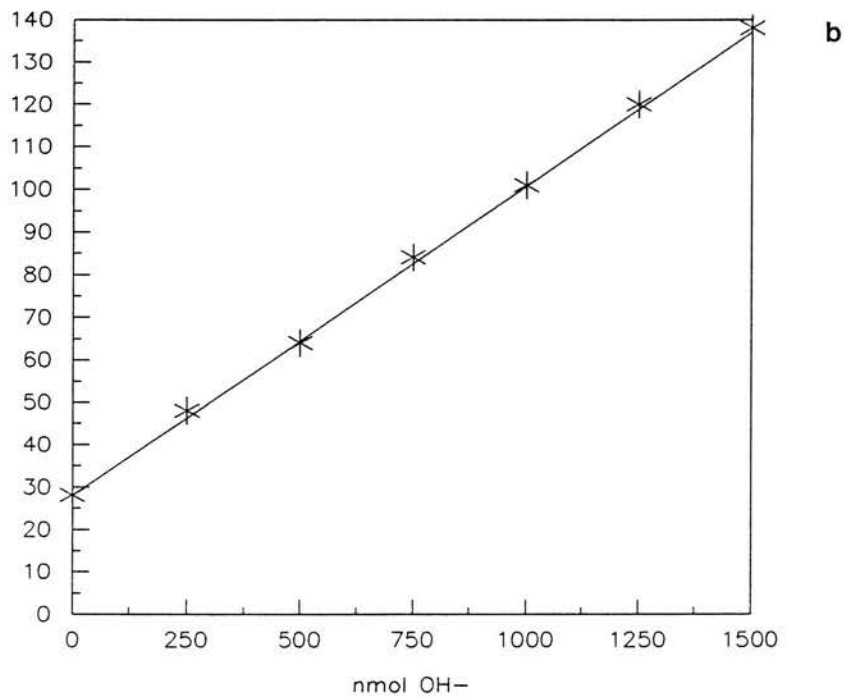
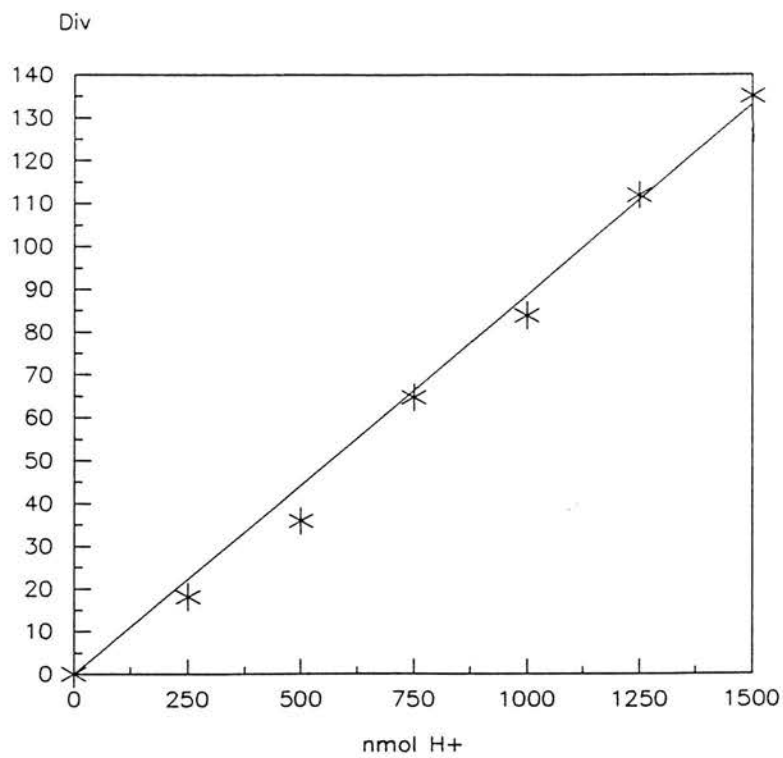


Figure 40. Calibration with HCl and KOH of the system for proton translocation experiments. The chamber contained 3mM HEPES buffer. A sensitive pH meter was connected to a chart recorder. The calibration was made from the deflection of the pen that occurred on adding 25ul aliquots of 0.01M HCl (Fig.40a) or 0.01M KOH (Fig.40b) which is equivalent to 250nmol H⁺ and OH⁻ respectively. The pH ranged from 7.14 to 6.88 during the calibration with HCl, and from 6.88 to 7.08 during calibration with KOH.

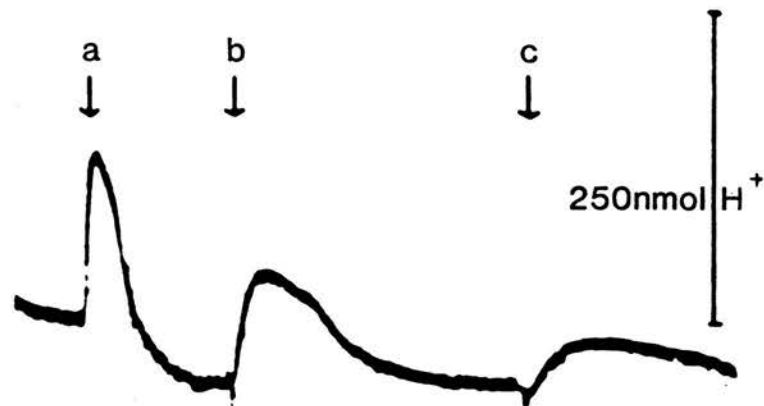


Figure 41. Proton translocation by rat liver mitochondria. The system was anaerobic, stirred and maintained at 27°C. The medium contained 125mM sucrose/60mM KCl/3mM HEPES/0.4mM Na succinate/ 6uM rotenone/0.4mM NEM/3.5uM valinomycin, pH7.2. 200ul rat liver mitochondria were added after anaerobic conditions were achieved. 150ul of air saturated buffer (equivalent to 39nmol O₂) were added at a, b and c.

Proton translocation by *C.mucosalis*

Proton translocation experiments with *C.mucosalis* were performed using whole fresh cells grown on serum agar plates for 1 day. The cells were harvested under N₂ into KCl/Hepes buffer, washed once only and suspended by gentle homogenisation in the same buffer. As whole cells, rather than spheroplasts, were used then the inclusion of sucrose as an osmotic support was not required. [In any case there was some indication (see section I), that sucrose might pre-dispose *C.mucosalis* cells to lysis and was therefore omitted to maintain the integrity of the membranes.]

The oxidation of endogenous substrates was shown (Figure 42a) not to result in any detectable proton translocation. Starvation, to exhaust substrates prior to experimentation, which was reported to be necessary with certain bacteria (Dawson and Jones 1981a), was not required for experiments with *C.mucosalis*. Rotenone was neither required nor desirable, as its presence may inhibit oxidation of exogenous as well as endogenous substrates.

With mitochondria, the inclusion of NEM increased the H⁺/O quotient (Brand et al. 1976) but with a methylotrophic bacterium, NEM decreased the quotient (Dawson and Jones 1981a). NEM was omitted from the *C.mucosalis* medium as there was no evidence for the presence of an NEM-sensitive H⁺/phosphate symporter.

Hydrogen or formate were used as the respiratory substrates for *C.mucosalis*. The traces (Figure 42c) show the results with H₂ (0.78mM) as substrate, which was supplied by flushing the chamber with H₂ gas in place of N₂. The addition of pulses of O₂ (39nmol) resulted in the generation of a proton gradient as measured by acidification of the external medium. Similar levels of acidification were observed when H₂O₂ (88nmol) was added in air-free buffer, as e⁻ acceptor in place of O₂. It should be noted that the addition of H₂O₂ to catalase-positive bacteria, or to systems containing exogenous catalase, would be equivalent to the addition of O₂ which

would be the result of the dismutation reaction.

Catalase-negative C.mucosalis was grown on catalase-free media, to prevent contaminating the system with this highly active enzyme. H_2O_2 added to C.mucosalis will not therefore be converted to O_2 , but will be reduced to H_2O by the action of cytochrome-c peroxidase.

The acidification that resulted from the addition of O_2 or H_2O_2 was not an artifact of the buffer, as de-oxygenated (N_2 gassed) buffer produced a minimal response (Figure 42c), which was probably due to residual traces of O_2 .

The acidification that occurred in response to the addition of e^- acceptors was abolished in the presence of the protonophore carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), but was only slightly inhibited by the respiratory inhibitor HOQNO (Figure 42c). This tends to confirm the previous observation (section IIb) indicating that this quinol analogue was only partially blocking e^- flow through the respiratory chain.

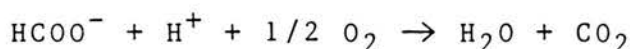
In the anaerobic state, when formate (1mM Na formate) was present as the respiratory substrate, there was a continual drift towards alkalinisation of the external medium (Figure 42b). This may represent H^+ entry into the bacterium, linked to the uptake of formate as uncharged formic acid (Garland et al. 1975). Thus :-



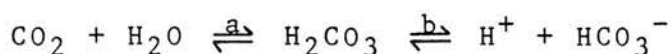
On adding the e^- acceptors O_2 or H_2O_2 to C.mucosalis, transient acidification of the external medium was observed (Figure 42b). The acidification achieved was likely to underestimate the level of H^+ translocation that occurred due to the alkalinisation resulting from the proton-linked formate uptake.

Changes to the pH may also result from the products of formate oxidation. CO_2 was reported to be the product of

formate oxidation (Kroger 1977)



CO₂ then undergoes the reaction (Garland et al. 1975) :-



Reaction a is accelerated by carbonic anhydrase. Reaction b has a pK of 6.1 and will be instantaneous (Garland et al. 1975). With Wolinella succinogenes (Kroger 1975), carbonic anhydrase-catalysed hydration of CO₂ compensated for the pH changes due to formate uptake and therefore enabled electron transport-driven proton translocation to be measured without interference from substrate transport. Carbonic anhydrase was included in the buffer for C.mucosalis experiments.

Stoichiometry of proton translocation by C.mucosalis

The stoichiometry of H⁺ translocation was very low for both oxygen (circa 0.7 H⁺/O) and H₂O₂ (circa 0.6 H⁺/H₂O₂) in Figure 42c. Doubling the quantity of e⁻ acceptor added, to 78nmol O₂ or 176nmol H₂O₂ gave enhanced acidification (Figure 42c), but this was not in proportion to the increased levels of oxidant, as acidification was only enhanced by approximately 15% and 40% with O₂ and H₂O₂ respectively. Decreased H⁺/O quotients with high pulses of O₂ was also reported to occur in mitochondria (Mitchell and Moyle 1967).

The low stoichiometry of H⁺ translocation in C.mucosalis may be due to the fragile nature of the cell membranes, which was seen during attempts to form spheroplasts from this organism (section I). In proton extrusion experiments, only bacteria with intact membranes will be capable of acidifying the external medium by vectorial H⁺ efflux from the cytoplasm, yet all the membranes present, broken or otherwise will be consuming the added e⁻ acceptors. Due to the nature of the cell membrane, it is possible that only a small proportion of the cells remain intact and able to acidify the medium.

From the results it seems probable that e^- transport to either O_2 or H_2O_2 as terminal acceptor, leads to the generation of a proton gradient across the cell membrane in C.mucosalis. H_2O_2 was shown (section IIb) to be a product of formate oxidation. The removal of H_2O_2 , rather than operating solely as a detoxification process, may also contribute to energy conservation by proton-pumping as electrons are transported to cytochrome-c peroxidase. This mechanism should not operate in the presence of catalase, as this would remove H_2O_2 without the involvement of e^- transfer from the electron transport chain. C.mucosalis is itself catalase-negative, but some growth media used contain catalase, which might adversely affect this method of energy conservation and hence growth of C.mucosalis. This hypothesis was tested by comparing the growth of C.mucosalis in the presence and absence of catalase. These experiments will be described below (section IIIb).

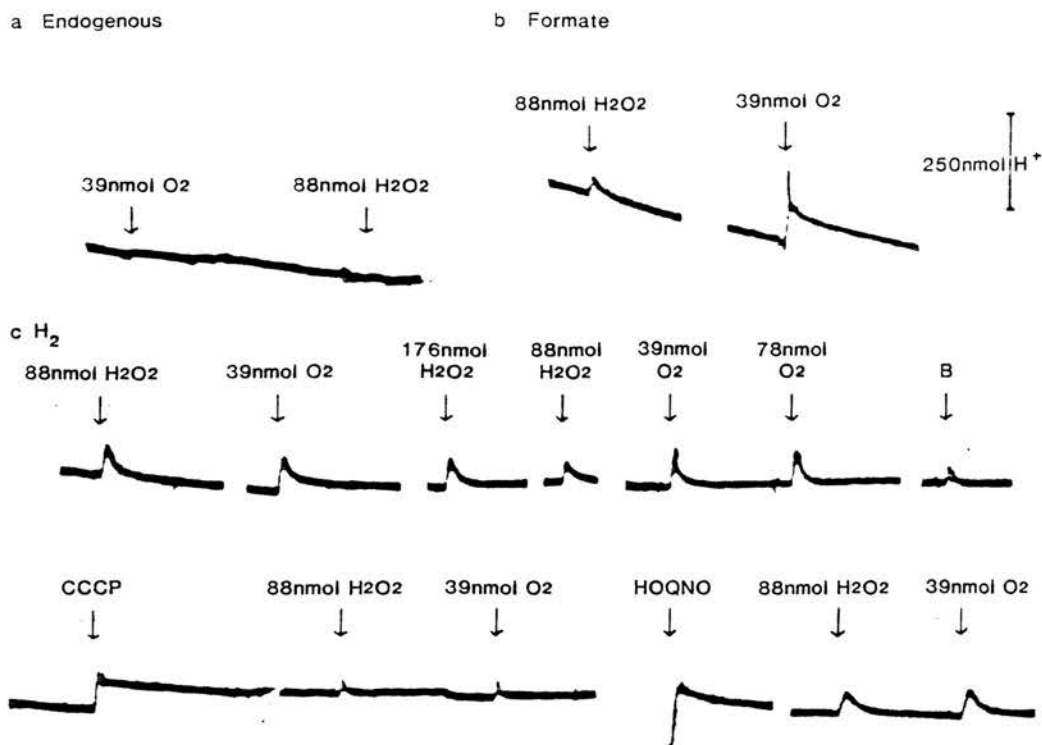


Figure 42. Proton translocation by *C. mucosalis*. The chamber was anaerobic, stirred, maintained at 27°C and contained 140mM KCl/3mM HEPES/200ug carbonic anhydrase/5uM valinomycin and 0.1ml fresh cell suspension (4mg protein) in a final volume of 3.5ml. The substrates were endogenous (a), 1mM formate (b) or 0.78mM H_2 (c), the latter being supplied by flushing the chamber with H_2 gas in place of N_2 . Acidification of the the medium was recorded following the addition of pulses of O_2 or H_2O_2 . Pulses of 39 and 78nmol O_2 were added by injection of 150 and 300ul air saturated buffer respectively. Pulses of 88 and 176nmol H_2O_2 were added in 10 and 20ul of O_2 -free solution respectively. 150ul of O_2 -free (N_2 gassed) buffer was added at B. 5uM CCCP or 30uM HOQNO were present as indicated.

IIIb The effect of catalase on the growth of *C.mucosalis*

1) **The determination of bacterial growth curves.**

Growth curves for bacteria grown in liquid culture can easily be determined by periodic sampling of the culture with absorbance measurements to estimate cell densities. Several attempts were made to grow *C.mucosalis* in liquid culture, microaerobically with H_2 as e^- donor, and anaerobically with thioglycollate to scavenge dissolved O_2 and formate and fumarate as e^- donor and acceptor respectively. Supplements of CO_2 , in the form of bicarbonate, and the addition of serum or other blood extract products, did not noticeably enhance the growth of *C.mucosalis*, which grew very poorly in all liquid culture media investigated. Growth curves for *C.mucosalis* could not therefore be derived from optical density measurements of liquid culture media. As an alternative method, cell yields from supplemented agar plates were used to determine the effect of catalase on the growth of *C.mucosalis*.

2) **Preparation of growth media with and without catalase**

Columbia agar (Oxoid) plates supplemented with 5% whole horse blood (CBA) contain erythrocyte catalase, serum agar plates should be free of this enzyme. Catalase estimations were made (Sigma Method) on a sample of horse blood used to prepare blood agar plates, which gave values of around 400 to 500 Sigma units per millilitre of medium. As each plate contains approximately 20ml media, then this is equivalent to 8000 to 10,000 Sigma units of catalase per blood agar plate. 1 Sigma unit decomposes 1.0umol H_2O_2 per minute at pH7 and 25°C. Therefore the capacity for CBA plates to dispose of H_2O_2 could be up to 10mmol per minute. The H_2O_2 -producing *C.mucosalis* grows only on the surface of the medium, therefore the actual maximum rate of peroxide removal will be lower than 10mmol per minute. However, significant levels of H_2O_2 removal by catalase at the surface of the medium is likely to occur. This was demonstrated by the vigorous evolution of O_2 that occurred

with the addition of a drop of 30% w/v (8.8M) H_2O_2 to the surface of a CBA plate. Agar plates supplemented with 5% horse serum in place of horse blood, gave no such reaction with H_2O_2 , thus confirming the absence of catalase in that medium.

Serum agar plates were also prepared supplemented with catalase. A solution of catalase was first sterilised by passage through a 0.22 μ m Millipore filter, and the enzyme activity was determined as before. 6000 Sigma units were spread over the surface of each plate, which was previously air-dried to facilitate absorption of the enzyme solution.

3) Growth of C.mucosalis in the presence and absence of catalase

C.mucosalis was subcultured onto agar plates supplemented with either horse blood, serum or serum plus catalase, and grown microaerobically (initially 4.5% O_2) with H_2 as e^- donor. At 1,2,3 and 4 day intervals the cells from one of the jars (which contained 12 plates i.e 4 of each type of media) were harvested. All the cells harvested from the 4 plates were suspended in buffer to a final volume of 20ml. Cell growths were estimated from A_{600} measurements of a 10 fold dilution (Figure 43a) and from wet weights after centrifugation (Figure 43b). Both methods gave similar results.

Cell growth was not greatly affected either by the type of media used, or by increasing the growth period (Figure 43), although the small sample size may mask the changes. The effect of catalase upon the growth of C.mucosalis was not clear from these results, although it appears that maximum growth was attained after 1 day.

To increase the sample size, C.mucosalis was then grown for 1 day on agar plates supplemented with either blood or serum. 24 of each type of plate were used, placed alternately in the microaerobic jars to equalise the atmospheric conditions experienced. In this experiment, the cell yield of C.mucosalis grown on serum was nearly twice (179%) that obtained on CBA plates.

a

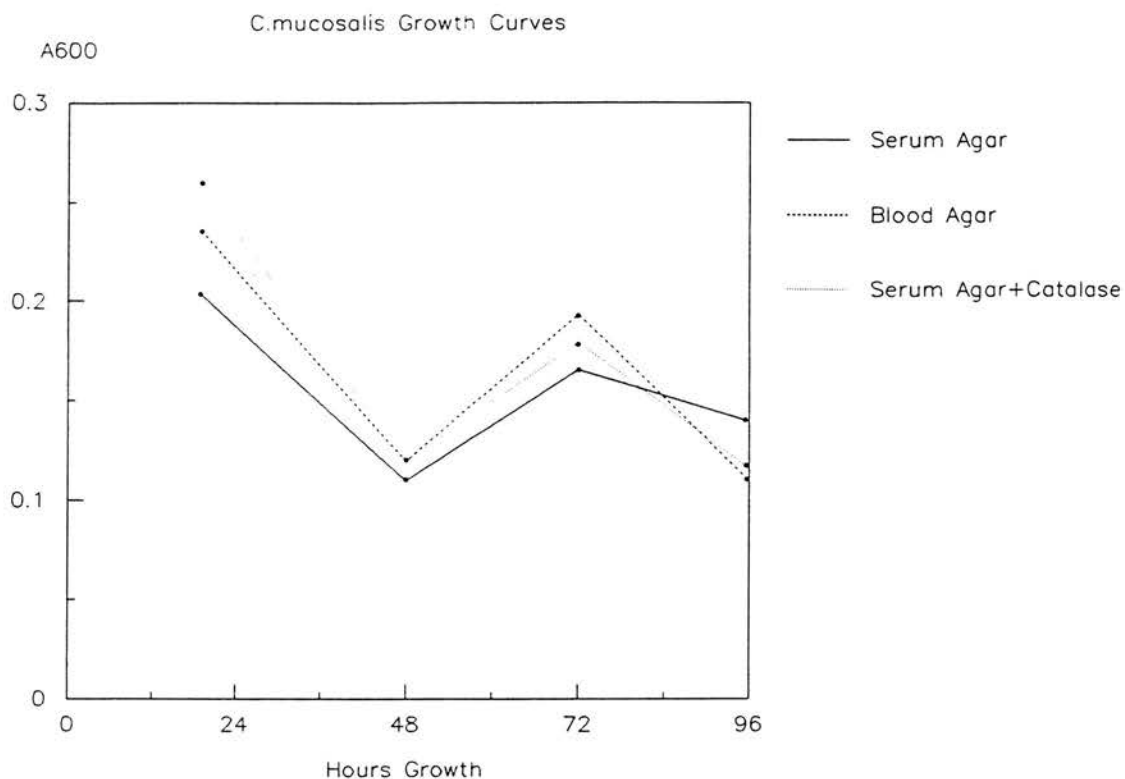
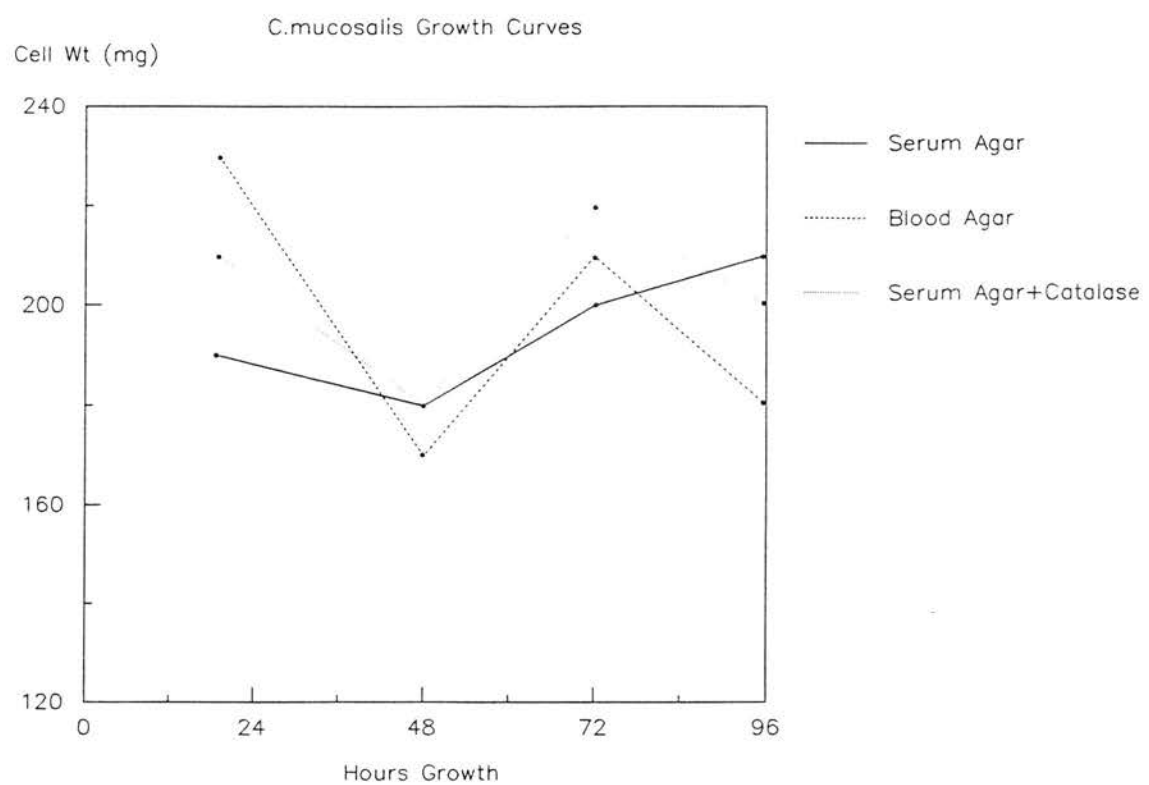


Figure 43. Growth curves for *C.mucosalis* in the presence and absence of erythrocyte catalase. *C.mucosalis* was grown on 4 agar plates supplemented with 5% horse blood, 5% horse serum or 5% horse serum plus 6000 Sigma units of catalase per plate for 19, 48, 72 and 96 hours. Cells from these 4 plates were harvested and suspended in 20ml phosphate buffered saline. Growth curves were made from the A₆₀₀ of a 10-fold dilution of the cell suspensions (Fig.43a), or from cell wet weights derived after centrifugation of the cell suspensions (Fig.43b).

b



Conclusions

From the results there was an indication that C.mucosalis may grow faster in the absence of catalase, thus supporting the hypothesis of an energy conserving mechanism operating with H_2O_2 as terminal e^- acceptor.

Failure to consistently elicit an effect with exogenous catalase may reflect problems of enzyme gaining access to the substrate, or may be due to bacterial adaptation in the presence of catalase. These alternative explanations will be discussed below.

The cytoplasmic membrane is impermeable to almost all solutes unless special transport systems are provided, whereas the outer membrane has limited permeability to small solutes via substrate-nonspecific proteinaceous channels called porins (Ames 1986). The exclusion limit for solute entry through the outer membrane of C.mucosalis is not known, but in E.coli and Ps.aeruginosa the entry of molecules above M_r 1000 was restricted (Caulcott et al. 1984), and soluble cytochrome c-553 with M_r 9000 is retained by intact C.mucosalis. Catalase, with a M_r of over 200,000, will therefore not pass through the outer membrane of bacteria. Any H_2O_2 produced by C.mucosalis will have to cross the outer membrane (and the cytoplasmic membrane if the site of production is cytoplasmic rather than periplasmic) before it can react with catalase. Cytochrome-c peroxidase, may still obtain supplies of H_2O_2 , even in the presence of high levels of exogenous catalase. Therefore catalase in the growth medium may not necessarily preclude the operation of energy conserving e^- transfer to the terminal acceptor H_2O_2 .

An alternative explanation involves bacterial adaptation to the presence of catalase in the growth medium. Removal of H_2O_2 by catalase may repress the e^- pathway to H_2O_2 and induce the alternative pathway utilising O_2 as acceptor. The presence of catalase in the growth medium was shown to repress the activity of cytochrome-c peroxidase (Chapter 3 section 3c) and to induce the activity of cytochrome-c oxidase (Chapter 4

section 2e).

Thus the effect of catalase may be to inhibit energy conservation utilising H_2O_2 as e^- acceptor and/or induce the operation of an e^- transfer pathway that does not involve this terminal acceptor.

Chapter 7

Model of microaerobic respiration in *C.mucosalis*.

Introduction

In this chapter a model for the microaerobic respiration of *C.mucosalis* will be proposed, based on the results presented in the previous 4 chapters. The results obtained are summarised below, to aid the discussion of the model. The model includes adaptation to growth conditions and a respiratory role for H_2O_2 . The catalase status and microaerophilic nature of *C.mucosalis* and related species will be discussed with reference to this novel form of energy conservation.

Summary of Results of the *C.mucosalis* experiments.

Cytochrome c-553 and soluble cytochrome-c peroxidase were induced while cytochrome-c oxidase activity was repressed in young cells in response to relatively high oxygen concentrations. Cytochrome-c oxidase activity was induced while cytochrome-c peroxidase activity and cytochrome c-553 levels were repressed in older cells when the O_2 concentration was lower.

Cytochrome-c-553 and cytochrome-c peroxidase were induced by the absence of erythrocyte catalase from the media or when formate as opposed to H_2 was present as e^- donor. Cytochrome-c oxidase was induced by the presence of erythrocyte catalase in the media or when H_2 rather than formate was present as e^- donor.

The reactivity of cytochrome c-553 with the *C.mucosalis* peroxidase and oxidase, and the ionic strength dependence of the cytochrome-c:cytochrome-c peroxidase reaction were consistent with the role of c-553 operating as the physiological e^- donor to both of these enzymes.

A high level of cytochrome-c peroxidase activity was

soluble, but membrane-associated peroxidase activity could not be determined. The soluble cytochrome-c peroxidase was unstable in the presence of H_2O_2 derived endogenously or from exogenous sources. The peroxidase was partially purified and has a native M_r similar to that of the Ps.aeruginosa cytochrome-c peroxidase.

The cytochrome-c oxidase was exclusively membrane-bound and exhibited a high affinity for O_2 . The activity of the oxidase was stable unless stored at $37^\circ C$. The oxidase could not be assigned into any of the classical bacterial terminal oxidase types on the basis of spectral data.

Formate dehydrogenase and formate oxidase activities were confined to the membrane fraction. FDH and formate oxidase activities were high in cells grown in the absence of formate, thus indicating that they are constitutive enzymes. The oxidation of formate involved an oxidase with a high affinity for O_2 .

The consumption of oxygen in the presence of formate resulted in the production of H_2O_2 and H_2O . H_2O_2 accumulation was enhanced, particularly in young cells, when the oxidation of formate was carried out in the presence of the respiratory inhibitor HOQNO. In the absence of inhibitors, H_2O_2 was removed by means of the cytochrome-c peroxidase.

A proton gradient with lower external pH was developed with H_2 or formate as e^- donors and either O_2 or H_2O_2 as the terminal electron acceptors.

Growth of C.mucosalis was sometimes measurably faster when erythrocyte catalase was absent from the growth medium.

Model of microaerophilic respiration in C.mucosalis.

The results summarised above were used to derive the proposed model shown in Figure 1b. The model has both the H_2O_2 produced from the oxidation of formate and H_2O_2 removal by the action of cytochrome-c peroxidase located in the periplasm. The reduction of O_2 to form H_2O is shown to occur on the cytoplasmic side of the membrane with the electrons supplied by transmembrane transport. The topography of bacterial

respiratory enzymes is generally determined following the production of spheroplasts. This was not possible with C.mucosalis as described in chapter 6 section I. The probable location of these enzymes can, however, be deduced from data derived from other bacterial species.

In the related species Wolinella succinogenes, formate dehydrogenase activity was found to be located on the periplasmic-facing side of the cytoplasmic membrane (Kroger 1977). The oxidation of simple reductants, such as H_2 and formate, was generally found to occur in the extra-cytoplasmic (periplasmic) compartment, releasing H^+ externally thus contributing to the formation of a useful proton gradient (Hooper and Dispirito 1985).

All known c-type cytochromes are either periplasmic or periplasmic-facing (Wood 1983). In C.mucosalis, soluble cytochrome c-553 is the probable physiological e^- donor to soluble cytochrome-c peroxidase. As the cytochrome-c is periplasmic, then the cytochrome-c peroxidase that reacts with it must also be located in the periplasm.

Cytochrome c-553 also reacts with cytochrome-c oxidase. This enzyme is most likely to be transmembrane (Harold 1972), with e^- transport leading to the reduction of O_2 on the cytoplasmic side of the membrane, and the associated proton uptake adding to the proton gradient.

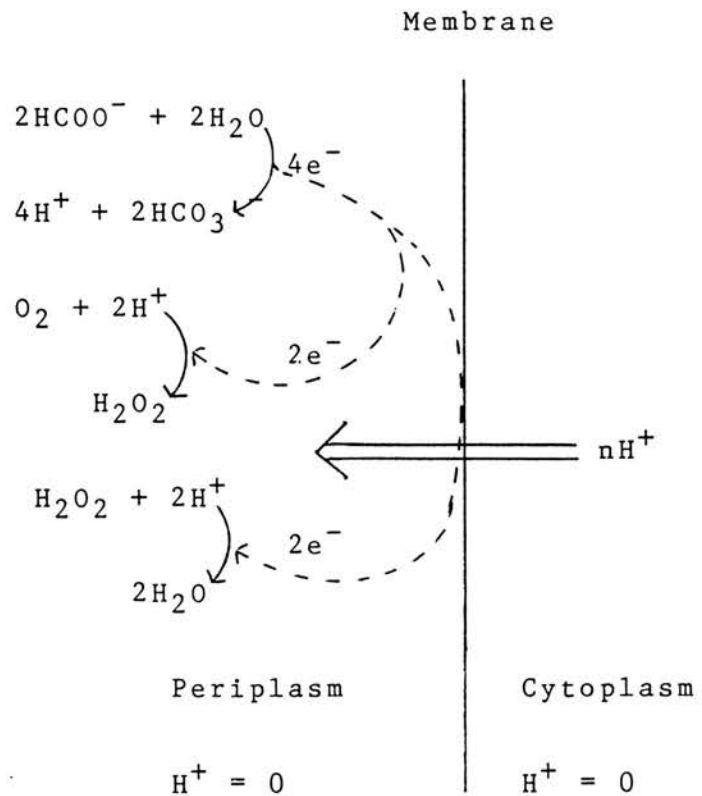
The different routes of e^- transfer defined in the model will be considered individually. These are 1) the oxidation of formate to form H_2O_2 and its subsequent removal by the action of cytochrome-c peroxidase and 2) the direct oxidation of formate to form H_2O .

1) Formate oxidation with the production and removal of H_2O_2 .

If H_2O_2 production as well as its removal is periplasmic, then proton release and uptake balance when formate is oxidised via H_2O_2 , as in the following diagram.

Figure 44

Model for energy conservation during the oxidation of formate that involves the production and removal of peroxide.



Therefore there is no net generation of a proton gradient by means of the reactions themselves. If, however, the oxidation of formate was cytoplasmic then the reactions would result in a net acidification of the cytoplasm and alkalinisation outside. Thus the generation of a bioenergetically useful acidification of the periplasm must result from protons pumped from the cytoplasm in response to e^- flow through the electron transport system. This is

indicated in the diagram by the transmembrane arrow.

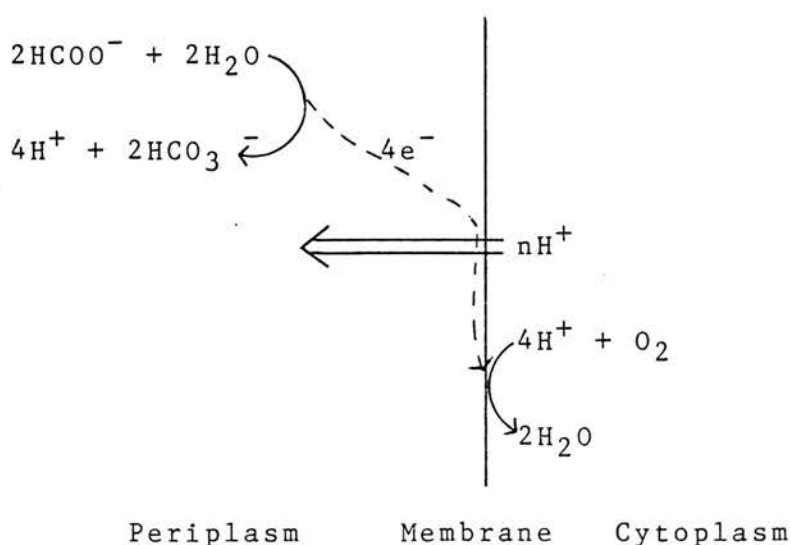
Proton translocation across the membrane in response to e^- flow to H_2O_2 as terminal acceptor was demonstrated by proton pumping experiments (chapter 6 section III). In this case, the system was anaerobic therefore H_2O_2 production from the reduction of O_2 did not occur. All $4e^-$ from the 2 formate molecules pass through the electron transport system, reducing 2 molecules of H_2O_2 to water. Hence $4H^+$ are released and $4H^+$ are taken up, and again the protons balance. The acidification of the external medium that was detected in response to a pulse of H_2O_2 (Figure 42b) must therefore be the result of protons translocated across the membrane as e^- were transported through the respiratory chain.

2) The direct oxidation of formate to H_2O .

The oxidation of formate directly to H_2O is likely to involve the release of protons into the periplasm and the uptake of H^+ from the cytoplasm (diagram 45 below).

Figure 45

Model for energy conservation during the oxidation of formate directly to water.



In this case, the reactions themselves result in a net

acidification of the periplasm without the need for proton translocation. If, as was indicated by the reactivity experiments (chapter 4), c-553 is the physiological e^- donor to the oxidase and the peroxidase, therefore the path of e^- flow from formate to either O_2 or H_2O_2 as terminal acceptors, will branch at the level of cytochrome c-553 (as in figure 1b). It seems likely that e^- flow to the peroxidase and to the oxidase results in proton translocation. The ratio of H^+/O should however be higher than the H^+/H_2O_2 ratio, to reflect the additional H^+ gradient generated by the favorable sidedness of the cytochrome-c oxidase reaction (Figure 45). The H^+/O ratio was slightly higher at 0.7, compared to 0.6 for the H^+/H_2O_2 ratio. The similarity of the results obtained with the two different e^- acceptors may indicate that the O_2 pulse was reduced to form H_2O_2 in preference to solely acting as e^- acceptor for the oxidase reaction.

The low stoichiometry of H^+ translocation means that the proportion of e^- flowing via the different routes cannot be determined. It seems probable, however, that the removal of H_2O_2 involving e^- flow through a proton-pumping respiratory chain contributes to energy conservation in C.mucosalis and is not just a detoxification mechanism.

Thus the proposed model (Figure 1b) involves alternative routes of e^- transfer involving either a branched pathway with oxidation of formate to form H_2O_2 followed by its peroxidative removal or a direct path of e^- transfer to reduce O_2 to H_2O . The proportion of e^- flow through these pathways could not be ascertained directly, but there was evidence (discussed below) that the growth conditions induced bacterial adaptations thus changing the proportion of formate that was oxidised via the intermediate H_2O_2 . These adaptations involved the induction and repression of respiratory chain components in response to the O_2 concentration, the e^- donor used and whether or not growth was in the presence of catalase.

In organisms that grow in liquid culture, the O_2 concentrations in the growth media can be maintained at a stable level, increased or decreased at will. Sampling and

assays of the bacterial culture can be made to determine the effects of d.o.t. on the respiratory enzymes. C.mucosalis does not grow on such media and was grown on agar plates in sealed culture jars. These jars initially contained O_2 at 20% of the atmospheric level, which gradually fell with time due to cellular respiration. The fall in O_2 concentrations was accompanied by repression of c-553 and cytochrome-c peroxidase and induction of cytochrome-c oxidase. This indicates a change in the nature of respiration. The proposal is that in early stages of growth, when oxygen concentrations are relatively high, H_2O_2 is produced from the oxidation of formate and then reduced by the action of the cytochrome-c peroxidase. As the O_2 concentration falls, less H_2O_2 is produced and e^- flow occurs via a conventional linear path to the cytochrome-c oxidase. High levels of H_2O_2 were not seen to accumulate in cells harvested early in growth, presumably due to the elevated levels of the H_2O_2 -removing peroxidase and its e^- donor c-553. Inhibition by HOQNO of the e^- flow through the electron transport system, restricts the supplies of reduced cytochrome-c and hence results in decreased activity of the peroxidase and the accumulation of high levels of H_2O_2 . The capacity for H_2O_2 production was shown to be higher in young cells than in cells harvested at a later stage in their growth. [In a recent paper (Ohta and Gottschal 1988) Wolinella recta grown at high d.o.t. were shown to produce more H_2O_2 than did cells grown at a lower d.o.t.]

As the O_2 concentration fell, induction of cytochrome oxidase and the repression of c-553 and the peroxidase occurred, consistent with utilisation in the older cells of the alternative route of e^- transfer to the oxidase. The repression of c-553, which is also the e^- donor to the oxidase, may reflect a lower requirement for cytochrome-c to effectively mediate e^- transfer between membrane complexes as compared to the high levels required to mediate between a membrane complex and a soluble peroxidase. The production of H_2O_2 and subsequent damage to enzymes has been implicated as a cause of oxygen toxicity in

microaerophilic bacteria. H_2O_2 can be generated by the direct interaction of autooxidisable dehydrogenases with O_2 (Krieg and Hoffman 1986). Formate dehydrogenase was shown to produce H_2O_2 in C.mucosalis (this work), C.sputorum (Niekus et al. 1980a, 1980b) and in C.jejuni (Hoffman et al 1979 a). C.mucosalis has been shown to be able to adapt to relatively high O_2 concentrations and to the presence of formate by the induction of cytochrome-c peroxidase. However, the microaerobic nature of C.mucosalis indicates that induction of cytochrome-c peroxidase activity is insufficient to allow growth under atmospheric levels of O_2 . It is interesting to speculate on the causes of this oxygen toxicity. It may be that the dehydrogenases of microaerophilic bacteria ^{also reduce O_2 and} have a high K_m for oxygen, as was found in C.sputorum (Niekus et al. 1980a). H_2O_2 production would therefore increase as the O_2 concentration was raised, and a point may be reached when the rate of H_2O_2 production exceeds its removal. The accumulation of this toxic product may result in irreparable damage occurring to enzyme systems. High susceptibility to damage has been reported for some anaerobic and microaerobic organisms (Krieg and Hoffman 1986).

The absence of catalase in C.mucosalis and C.sputorum would seem likely to make these organisms more susceptible to damage by H_2O_2 than are the catalase positive species. The addition of catalase to the growth media of C.mucosalis and C.sputorum, however, does not result in enhanced aerotolerance. Some microaerophiles do exhibit enhanced aerotolerance when catalase is present in the media and C.fetus (Hoffman and Goodman 1982) can even grow under atmospheric levels of O_2 when catalase and an O_2^- scavenging medium are used. As discussed in chapter 6 section IIIb, catalase in the growth medium of C.mucosalis may fail to gain access to the substrate and therefore it would be unable to enhance the aerotolerance of the organism. This may explain why certain bacteria have been found that possess both catalase and peroxidase activities. It was suggested (Goodhew et al. 1988) that such seeming duplication may facilitate the

efficient scavenging of peroxide in two separate cellular compartments, with catalase and peroxidase located in the cytoplasm and periplasm respectively. Each scavenger would thus remove the H_2O_2 produced in their own compartments. The catalase and cytochrome-c peroxidase of Ps.stutzeri and Ps.aeruginosa have been shown to be so located (Wilson, Goodhew, Hunter and Pettigrew, unpublished data). An alternative explanation is that peroxidase is important as a scavenger for low levels of H_2O_2 , as catalase has a low affinity for H_2O_2 (Krieg and Hoffman 1986).

In C.mucosalis, the presence of catalase in the media was shown to affect the growth of the organism, which suggests that catalase was gaining at least limited access to its substrate. Unlike in most organisms, the effect of catalase was to inhibit growth of C.mucosalis. The explanation for this may be that the presence of catalase not only removes toxic H_2O_2 , but also prevents the operation of the proton translocating electron transport chain supplying e^- to the peroxidase. The presence of catalase thus deprives the organism of this method of energy conservation. The catalase-negative nature of C.mucosalis and C.sputorum therefore allows these organisms to conserve energy using the H_2O_2 that seems to be an inevitable product of formate oxidation. This mechanism may not be available to catalase positive organisms that, on exposure to high oxygen levels, will produce H_2O_2 which will be detoxified without any gain to the organism, and which is also wasteful of the substrate material.

The environment in which C.mucosalis normally resides (the intestine) will maintain microaerobic conditions with only small variations in the oxygen tension. The risk of exposure to atmospheric oxygen tensions would seem to be remote. Adaptations that allow energy conservation to occur with both H_2O_2 and O_2 as electron acceptors would allow efficient utilisation of the substrates available to the organism in its physiological environment. In conclusion, production of H_2O_2 , though unavoidable in this organism, has

it seems been exploited to play a positive role in energy conservation.

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Haem staining in gels, a useful tool in the study of bacterial *c*-type cytochromes

C.F. Goodhew, K.R. Brown and G.W. Pettigrew

Biochemistry Unit, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh (U.K.)

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c-Type cytochromes are the only cytochromes to retain quantitatively their haem during SDS gel electrophoresis and can be identified in complex mixtures by their haem peroxidase activity. Although weak staining bands may be due to residual haem attachment to *b*-type cytochromes or to migration of haem, these effects could be abolished by prior extraction with organic solvent. The colour yield of haem staining allowed an estimate of the relative amounts of a particular cytochrome, particularly if loadings were below 50 pmol. At greater loadings, a plateau of colour development was observed. Freshly made gels gave much poorer colour development. The haem-staining method was shown to be useful in three particular areas of study in bacterial respiration. Firstly, it allows assessment of the results of sphaeroplast formation in gram negative bacteria. Secondly, quantitation of the haem stain was useful in the investigation of the induction effects of growth conditions on *c*-type cytochromes. Thirdly, the interpretation of complex chromatographic profiles was greatly simplified by the use of haem-stained SDS electrophoretic gels.

Introduction

Proteins retaining haem in SDS can be detected by their haem peroxidase activity after electrophoresis using 3,3',5,5'-tetramethylbenzidine (TMBZ) as an oxidisable substrate which gives rise to a blue insoluble precipitate [1]. The method was developed, and has been widely used, for proteins such as cytochrome *P*-450 and cytochromes *b* which contain protohaem IX [1–3], but because this haem is not covalently bound, only a residue is retained under denaturing conditions and this varies depending on the precise conditions employed.

On the other hand, the *c*-type cytochromes contain covalently bound haem and are ideally

suited to the application of the peroxidase activity method after SDS electrophoresis, yet it has been relatively little used in this context (a notable exception is the paper of Ward et al. [4]).

In our laboratory the haem-staining method has become a routine and invaluable method in three particular areas in our studies of bacterial respiration. These are the assessment of sphaeroplast formation, the investigation of induction of *c*-type cytochromes and the identification of different *c*-type cytochromes in complex chromatographic profiles. In view of a recent paper [5] pointing out a potential shortcoming of the method, we thought it appropriate to present a paper emphasising the considerable merits of the method and commenting on the ways in which problems of interpretation can be solved.

Materials and Methods

Growth of cells and preparation of cell extracts
Pseudomonas stutzeri 224. Aerobic growth of

Abbreviation: TMBZ, 3,3',5,5'-tetramethylbenzidine.

Correspondence: Dr. G.W. Pettigrew, Biochemistry Unit, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh, U.K.

Pseudomonas stutzeri 224 (ATCC No. 17591) was done in a medium (pH 7) containing 17 mM tri-sodium citrate/7 mM KH_2PO_4 /2 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /4 g yeast extract (Oxoid) per l. 1-l cultures were incubated for 16 h, at 30°C on an orbital shaker. For denitrifying growth, NaNO_3 was added to a final concentration of 58 mM after 5 h of aerobic conditions, the flasks sealed with two layers of Nescofilm and further incubated at 30°C for 16 h. Cells were harvested by centrifugation at $4000 \times g$ for 30 min at 4°C (Beckman J-6B fitted with J.S 5.2 rotor) and washed once in 10 mM sodium phosphate (pH 7) buffer and re-centrifuged as above.

Periplasmic, cytoplasmic and membrane fractions were prepared by the method of Wood [6], except that cells were suspended to a final concentration of 100 mg/ml.

Acid-acetone and ethanol-acetone extracts. 10 vols. of 0.01 M HCl in acetone was added to the sample and after 30 min the precipitate was sedimented in a Beckman microfuge. The pellet was then dissolved in 62.5 mM Tris-HCl/2 mM EDTA/2% SDS/10% glycerol (pH 7), ready for electrophoresis.

Ethanol-acetone extraction was carried out similarly using ethanol/acetone (1:1).

SDS-polyacrylamide electrophoresis. Electrophoresis was carried out using the buffer system of Laemmli [7] with the addition of 2 mM EDTA.

Gels were cast (% acrylamide and % bis-acrylamide detailed in the figure legends) as slabs ($130 \times 150 \times 1$ mm) with a 4% acrylamide stacking gel, and run at 50 V for approx. 16 h, or at 150 V for 4–5 h.

Gels were stained for haem by the method of Thomas et al. [1] with the following modifications. Gels were immersed for 30 min in 1.25 mM TMBZ in methanol/0.25 M, sodium acetate (pH 5) (30:70) with constant shaking, after which the H_2O_2 was added to 26 mM. After shaking for a further 15 min the staining solution was replaced twice with fresh propanol/0.25 M sodium acetate (pH 5) (30:70). Photography and scanning of gels was carried out within 2 h of staining.

The centre of each channel was scanned using a Shimadzu CS-930 gel scanner at 690 nm in transmission mode with a beam of dimensions 0.05×2

mm in 0.2 mm steps. Photography of gels was through a yellow filter (Cokin A.001).

Gels were stained for protein with 2.0 g/l Coomassie brilliant blue R; C.1.42660 in methanol/acetic acid/water (4.5:1:4.5), destained in methanol/acetic acid/water (3:1:6) and photographed as above.

Ion-exchange chromatography. Samples were desalted into 5 mM Tris-HCl (pH 8) on Sephadex G-25 coarse and adsorbed onto DEAE cellulose (Whatman DE52, 10×1 cm) equilibrated with 5 mM Tris-HCl (pH 8). The column was developed with a 5 mM Tris-HCl to 5 mM Tris-HCl + 200 mM NaCl linear gradient (400 ml total volume).

Enzyme assays. Isocitrate dehydrogenase assays were carried out using the method of Bernt and Bergmeyer [8].

Results and Discussion

Assessment of the method

Specificity

The haem of *c*-type cytochromes is covalently bound and quantitatively retained on the protein in SDS. In contrast, most of the haem in proteins containing protohaem IX is dissociated under denaturing conditions and is observed as a rapidly migrating band exhibiting TMBZ peroxidase activity on SDS electrophoretic gels. However, a small and variable portion of protohaem IX is retained by such proteins. We have observed this with myoglobin, *Escherichia coli* cytochrome *b*-562 and yeast cytochrome *c* peroxidase (results not shown) and others have found the same for membrane-bound *b*-type cytochromes [2,3].

In addition to this residual retention of protohaem IX, there may be artefactual transfer to non-haem proteins. For example, ovalbumin will stain for TMBZ peroxidase activity if subjected to gel electrophoresis together with myoglobin (results not shown).

Both the residual retention of protohaem by myoglobin and its transfer to ovalbumin can be minimised or eliminated by prior treatment with organic solvents such as acidified acetone or ethanol/acetone (results not shown). The transfer of haem to ovalbumin can be blocked by treatment with *N*-ethylmaleimide implicating protein sulphydryl groups.

In a complex sample known to contain both *b*- and *c*-type cytochromes such as the bacterial membranes of Fig. 1, a pattern of haem staining will be obtained which cannot be interpreted without further information. The bands labelled a, b and c are not affected by treatment with *N*-ethylmaleimide or extraction with ethanol-acetone and can therefore be identified as *c*-type cytochromes. The bands labelled 1, 2 and 3 diminish or disappear with *N*-ethylmaleimide treatment or extraction with ethanol-acetone. These bands may represent *b*-type cytochromes with residual haem attachment, but the possibility of artefactual migration of protohaem IX cannot be excluded.

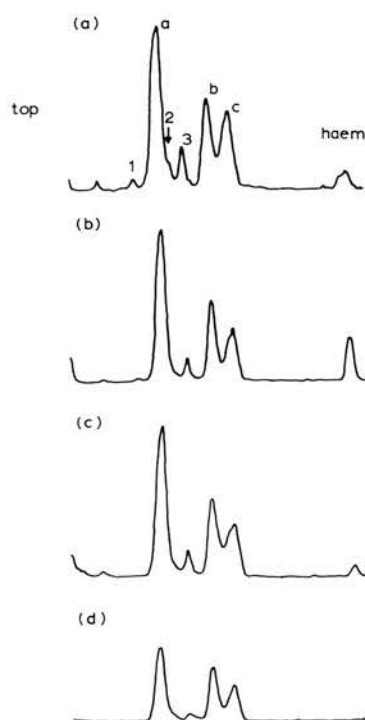


Fig. 1. Haem staining of membrane cytochromes from *Pseudomonas stutzeri*. Electrophoresis was performed in a gel containing 12.5% acrylamide and 0.3% bisacrylamide using spheroplast membrane suspensions of aerobically grown *Ps. stutzeri* 224. Equal amounts of membranes were loaded in each channel, except for (d) where the precipitate after acid-acetone treatment did not fully redissolve in SDS solution. (a) Untreated membranes; (b) membranes + 100 nmol *N*-ethylmaleimide; (c) membranes extracted with ethanol acetone; (d) membranes extracted with acid-acetone. Top indicates loading position of the vertical slab gel.

Extraction with acid-acetone also removes bands 1, 2 and 3 (Fig. 1d), but the protein precipitate dissolves poorly and overall decrease of all bands is observed.

With photosynthetic systems, a further complication is photooxidation of TMBZ by chlorophylls [3]. However, this can be avoided either by prior extraction with ethanol/acetone or by staining in the dark (results not shown).

Miller and Nicholas [5] found that a Cu-containing oxidase showed TMBZ oxidase activity under denaturing gel electrophoresis. Such an activity can easily be distinguished from haem peroxidase activity by examination of the gel before addition of H_2O_2 .

With these precautions and controls, electrophoretic bands with TMBZ peroxidase activity can be reliably identified as *c*-type cytochromes. Such an identification can be further confirmed by separation of the individual cytochromes and spectrophotometric characterisation.

Quantitation

Haem staining is a progressive, catalysed reaction rather than a stoichiometric process and this leads to problems of quantitation in a gel. However, the following experiments suggest that, with some precautions, the method can yield useful information on the relative amounts of *c*-type cytochrome present.

The data of Fig. 2 (solid lines) are for a single gel containing different amounts of two *c*-type cytochromes. Colour yield was measured after the standard staining procedure as described in the Materials and Methods. Several comments can be made. First, the two different cytochromes give similar colour yields. Second, the staining is sensitive, detecting 10 pmol of haem *c* with ease. Third, with amounts of haem greater than 50 pmol, a plateau of staining is observed. This may be due either to local exhaustion of TMBZ or H_2O_2 in the gel, although the effect was still observed with doubled concentrations (results not shown). Alternatively, this effect may be due to haem destruction by H_2O_2 . Fourth, the colour yield for 100 pmol or less, is much poorer in a freshly prepared gel (Fig. 2, broken lines), perhaps due to residual persulphate or derived peroxides either interfering directly with the haem-staining reaction or pro-

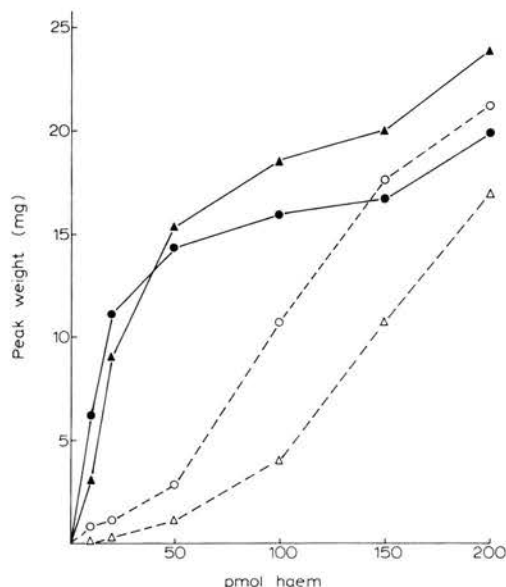


Fig. 2. The variation of colour yield with quantity of purified cytochromes in stored and fresh gels. Electrophoresis was performed in a gel containing 15% acrylamide and 0.4% bisacrylamide on samples containing various amounts of horse cytochrome *c* (●, ○) and *Pseudomonas aeruginosa* cytochrome *c*-551 (▲, △). In one experiment (closed symbols) a gel was used which had been allowed to 'age' for 18 hours; in a second experiment (open symbols) a freshly made gel was used. The central portion of each channel was scanned at 690 nm and the peaks cut from the chart paper and weighed.

gressively damaging the haem during electrophoresis.

As we will show in the following section one of the most powerful applications of the haem-staining method is the assessment of induction of bacterial *c*-type cytochromes under different growth conditions. Because of the plateau of haem staining, however, different amounts of a *c*-type cytochrome might give similar colour yields of staining. This problem can readily be overcome by ensuring that loadings lead to colour yields below the plateau region. Fig. 3 shows different loadings of periplasmic samples from aerobic and denitrifying *Pseudomonas stutzeri*. The colour yields for all the bands fall below the plateau region of Fig. 2 (the ordinates are comparable) and the differences in the levels of the cytochrome *cd*₁ and 30 K cytochrome *c* with the growth conditions are clearly seen at all loadings.

Some workers specifically avoid [1,3] or include

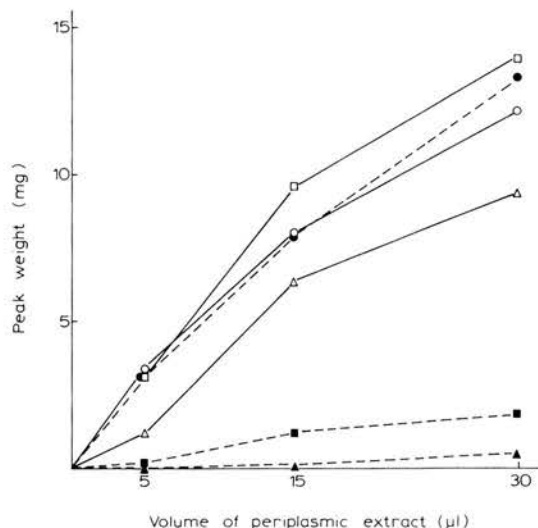


Fig. 3. The variation of colour yield with the quantity of periplasmic extract from *Pseudomonas stutzeri*. Electrophoresis was performed in a gradient gel of 10% acrylamide, 0.2% bisacrylamide to 25% acrylamide, 0.5% bisacrylamide on samples of periplasmic extracts of *Ps. stutzeri*. Individual cytochromes were identified with reference to purified markers and the colour yield associated with each band was expressed as a peak weight as described in Fig. 2. Cytochromes present in aerobically grown cells are denoted by closed symbols: those present in denitrifying cells by open symbols. Cytochrome *cd*₁ (nitrite reductase) (△, ▲); 30 K cytochrome *c* (□, ■); cytochrome *c*-551 (○, ●).

[2] reducing agents when running electrophoresis gels for haem staining. In our experience the presence of dithiothreitol leads to poor or negligible haem staining. This is probably due to dissociation of Fe II from the haem to yield a porphyrin and indeed this forms the basis for the fluorescence method of cytochrome *c* detection in electrophoretic gels [9,10].

Applications

Topography of bioenergetic membranes – sphaeroplast formation

The topography of redox centres on either side of, and within, a bioenergetic membrane is an important ingredient of the chemiosmotic explanation of energy conservation. In bacteria, Wood [11] has proposed that *c*-type cytochromes are located either in the periplasmic space or bound to the periplasmic side of the cell mem-

brane. Haem-stained gels of sphaeroplast fractions afford a good means of testing this proposal.

The electrophoretic analysis of the periplasmic, cytoplasmic and membrane-bound compartments of aerobic and denitrifying *Pseudomonas stutzeri* is shown in Fig. 4 and 5. It is clear from Fig. 4, channels 1a and b and 2a and b that the soluble cytochromes *c*-551, *cd*₁ and 30 K are located in the periplasmic space. The small percentage (6%) of cytochrome *c*-551 found in the cytoplasmic fraction may simply be due to entrapment in the sphaeroplast pellet or to partial sphaeroplast formation with incomplete release of periplasmic contents until osmotically shocked. The latter is the main problem associated with sphaeroplast studies in bacteria. In our hands, for example, *Pseudomonas aeruginosa* gives incomplete sphaeroplast formation and the 'cytoplasmic' fraction can contain as much as 80% of the cytochrome *c*-551. The problem is not always realised and has given rise to confusing proposals for the

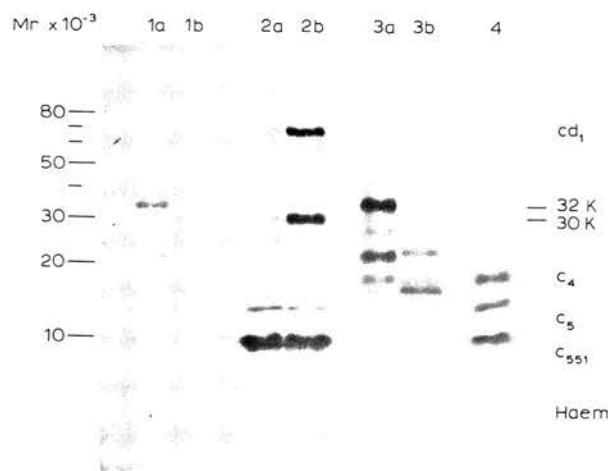


Fig. 4. Assessment of sphaeroplast formation: haem staining after SDS electrophoresis of cytoplasmic, periplasmic and membrane fractions of aerobic and denitrifying *Pseudomonas stutzeri*. The cytoplasmic (1a, 1b), periplasmic (2a, 2b) and membrane (3a, 3b) fractions were obtained from aerobic (1a, 2a, 3a) and denitrifying (1b, 2b, 3b) *Ps. stutzeri* as described in the Materials and Methods section. These fractions were adjusted to the same volume and equal portions were subjected to SDS electrophoresis as described in Fig. 3. Lane 4 contains 0.1 nmol of purified *c*₄, *c*₅ and *c*-551 from *Ps. stutzeri*. The scale of *M_r* was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

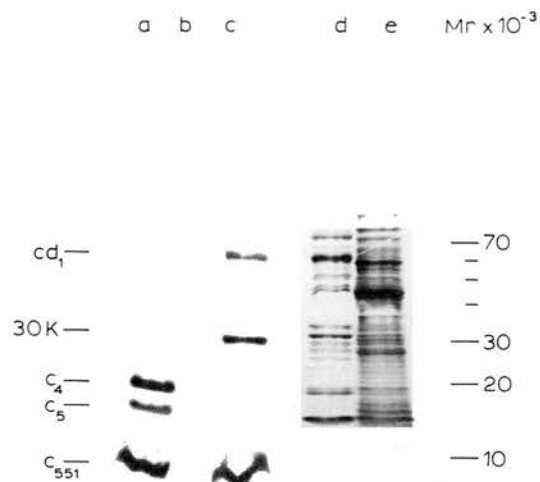


Fig. 5. Assessment of sphaeroplast formation: protein staining after SDS electrophoresis on a 5% acrylamide, 0.1% bis-acrylamide to 20% acrylamide, 0.5% bisacrylamide gradient gel of periplasmic and cytoplasmic fractions of denitrifying *Pseudomonas stutzeri*. Lane (a), 0.1 nmol of purified cytochromes *c*₄, *c*₅ and *c*-551; lanes (b) and (c): cytoplasmic fraction; lanes (c) and (d): periplasmic fraction. Lanes (a), (b) and (c) were stained for haem. Lanes (d) and (e) were stained for protein with the Coomassie blue reagent. The scale of *M_r* was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

dual location of, for example, cytochrome *c*₃ in *Desulfovibrio* [12].

The integrity of the cytoplasmic compartment after the first phase of the sphaeroplast formation cannot be assessed by haem-stained gels and requires the assay of a cytoplasmic enzyme marker. In the present studies only 2–4% of isocitrate dehydrogenase was released into the periplasmic fraction, indicating stability of the sphaeroplasts formed. The absence of cytoplasmic contamination of the periplasmic fraction and the relatively simple composition of the latter is shown by protein staining after electrophoresis (Fig. 5). There are only a few periplasmic proteins and the 30 K cytochrome and cytochrome *cd*₁ can be identified as dominant components. The small cytochrome *c*-551 probably leaches out during the prolonged staining and destaining procedure.

The membranes have their own distinctive complement of *c*-type cytochromes (Fig. 4) and the fast-migrating free haem indicates the prob-

able presence of *b*-type cytochromes also. There is perhaps more severe contamination (up to 15%) of the cytoplasmic fraction with membrane cytochromes than with soluble cytochromes and this is probably due to incomplete membrane sedimentation from the viscous DNA-containing sphaeroplast lysate.

Induction of *c*-type cytochromes

Bacteria are often bioenergetically versatile and can induce appropriate electron-transfer components in response to a changed environment. Most studies on the effects of growth conditions on cytochrome *c* (see, for example, Refs. 13 and 14) simply measure the change in total cytochrome *c* content. However, bacteria often contain several *c*-type cytochromes and individual trends will be lost in the total change. One solution is to purify individual cytochromes *c* (see for example Refs. 15 and 16), but this is time-consuming and differential losses during purification may give misleading results.

Haem-stained gels of soluble and membrane extracts allow examination of the individual cytochromes present in the whole system and, as indicated in section A, an estimate can be made of their relative amounts under different growth conditions. The periplasm of denitrifying *Ps. stutzeri* contains greatly increased amounts of cytochrome *cd*₁ (a nitrite reductase) and a 30 K cytochrome *c* (Fig. 3 and 4). In the membrane fraction a 32 K cytochrome *c* (which may correspond to the cytochrome *c*₁-like protein identified in *Ps. aeruginosa*) [17] is the dominant component of aerobic membranes but is almost absent from denitrifying membranes (Fig. 4).

Identification of cytochromes *c* during purification

As noted above, a bacterial extract will usually contain several *c*-type cytochromes with similar spectroscopic properties. During the initial stages of purification it is often difficult to keep track of the cytochrome of interest and we have found that haem staining after SDS electrophoresis to be a valuable method for assessment of the cytochrome composition of groups of chromatographic fractions.

A chromatographic separation of the periplasmic cytochromes of *Ps. stutzeri* is shown to

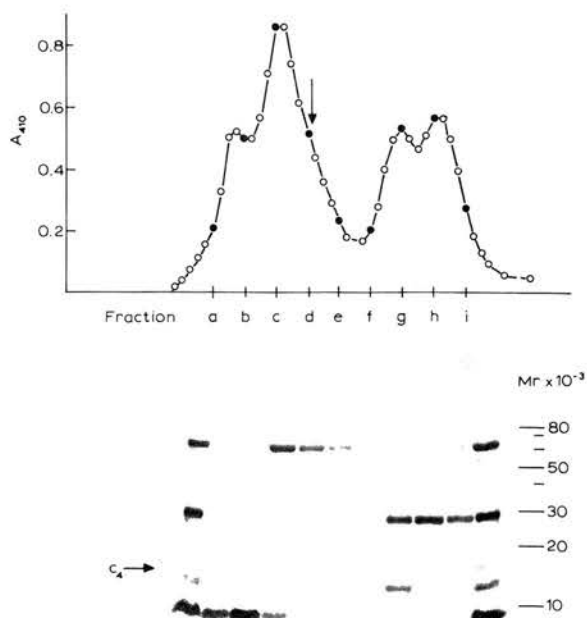


Fig. 6. Ion-exchange chromatography of the periplasmic fraction of denitrifying *Pseudomonas stutzeri*. Equal portions of every fourth fraction (designated a-i) eluted from a DEAE cellulose column were subjected to SDS gel electrophoresis as described in Fig. 3 and haem staining. The cytochrome *c* components of each fraction were compared with the total cytochrome *c* composition of the periplasmic fraction (outer lanes). The arrow indicates a shoulder on the trailing edge of the main chromatographic peak which is shown on the gel to be due to the presence of a small amount of cytochrome *c*₄. The scale of Mr was constructed from the relative mobilities of a set of molecular-weight marker proteins (not shown).

illustrate this point (Fig. 6). The periplasmic cytochromes were adsorbed to DEAE cellulose and eluted with a salt gradient. Equal portions of individual fractions were subjected to SDS electrophoresis treatment followed by haem staining (Fig. 6). Using the profile of A_{410} alone, the presence of overlapping peaks would make the choice of fractions to pool for further purification very difficult. With the aid of the gel, however, all the components of the periplasmic extract (outside lanes) can be detected in the chromatographic profile and fractions containing a particular cytochrome could be combined. For example, the minor component, cytochrome *c*₄, appears only as a slight shoulder in the chromatographic profile (indicated

by an arrow) but, by reference to the gel, its position in the profile can be clearly defined.

Acknowledgements

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The microaerophilic respiration of *Campylobacter mucosalis*

Celia. F. Goodhew, A.B. ElKurdi and Graham. W. Pettigrew

Biochemistry Unit, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh (U.K.)

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A model is proposed for the respiratory adaptation to falling oxygen concentration during growth of the microaerophilic bacterium *Campylobacter mucosalis*. During the early stages of growth, the oxidation of formate is a two-stage branched process involving the production of H_2O_2 followed by its peroxidatic removal. In later stages of growth, at lower oxygen concentrations, the predominant electron flow is linear to a membrane-bound cytochrome-*c* oxidase which reduces O_2 directly to H_2O . Several components of this model have been investigated. H_2O_2 was produced during formate oxidation and accumulated when electron transfer to the cytochrome-*c* peroxidase was inhibited. A cytochrome *c*-553, of the Class I type, was purified and shown to be the specific electron donor to both the peroxidase and the membrane bound oxidase. The levels of this cytochrome *c* and of the peroxidase were higher in cells harvested early in growth. In later stages of growth, the activity of the membrane-bound oxidase increased. Proton pumping across the membrane was detected with either H_2O_2 or oxygen as terminal electron acceptor. The novel energy-conserving role of H_2O_2 in this catalase-negative bacterium is discussed in relation to its microaerophilic nature.

Introduction

Microaerophilic bacteria utilise oxygen as a terminal electron acceptor in respiration, but are killed by high oxygen concentrations. This toxicity is probably due to the generation of partially reduced species, such as superoxide and hydrogen peroxide. The optimum oxygen tension for a particular microaerophile will be influenced by a number of factors, which include the rate of production of such toxic species, the susceptibility of

enzyme systems to damage, and the presence and efficiency of detoxifying enzymes such as superoxide dismutase, catalase and peroxidase [1].

The genus *Campylobacter* includes gram-negative, non-fermenting microaerophilic organisms [2]. Some, such as *C. sputorum* subspecies *bubulus* and *C. mucosalis*, are catalase-negative. In *C. sputorum* ss *bubulus*, Stouthamer and coworkers have proposed that hydrogen peroxide production during formate oxidation was a major cause of oxygen toxicity. The oxidation of formate occurred via two pathways. One, which was of low oxygen affinity, was inhibited by CN^- but not by quinol analogues, and produced hydrogen peroxide. The other, which was of high oxygen affinity, was inhibited by quinol analogues but not by CN^- and produced water as an end-product [3–5]. When the high affinity route was inhibited by glutaraldehyde treatment, hydrogen peroxide accumulated

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenyldiamine dihydrochloride; HOQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; CCCP, carbonylcyanide *m*-chlorophenylhydrazine.

Correspondence: C.F. Goodhew, Biochemistry Unit, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Summerhall, Edinburgh, U.K.

[6]. These authors propose that in uninhibited cells, hydrogen peroxide is decomposed by a cytochrome-*c* peroxidase supplied by the electron transport system. However the components of this electron transport system to the peroxidase were not investigated.

We studied the related catalase-negative organism *Campylobacter mucosalis* [7] originally isolated from porcine intestinal adenomatosis [8]. We found that this organism, like *C. sputorum* subspecies *bubulus*, produces substantial amounts of hydrogen peroxide during formate oxidation.

In this paper, we report a model for the respiration of *C. mucosalis* which involves an adaptation to changing oxygen concentrations during growth. Central to this model are the interactions of a soluble cytochrome *c*-553 with a soluble peroxidase and a membrane-bound cytochrome oxidase. In addition, we show that removal of hydrogen peroxide involves electron flow through an energy-conserving electron transport system.

Materials and Methods

Growth of cells

Campylobacter mucosalis f. *C. sputorum* subspecies *mucosalis* (Strain 1248, NCTC 253/72, [7]) was a gift from Dr. G.H.K. Lawson, [8] and was grown microaerobically on plates of Columbia agar base (Oxoid, Basingstoke, U.K.) supplemented with 5% horse serum or whole horse blood (Gibco, Paisley, U.K.) in an atmosphere initially comprising 4.5% O₂/5% CO₂/15.5% N₂/75% H₂. After growth at 37°C for 24 or 72 h, the cells were harvested by scraping from the agar surface and were washed in 10 mM sodium phosphate (pH 7).

Preparation of cell extracts and membranes

All operations were conducted at 4°C. Washed cells were homogenised in 3–5 vols. of 10 mM sodium phosphate (pH 7), and were disrupted either by freezing and thawing twice, or by passage through a French pressure cell at $82.7 \cdot 10^6$ Pascals (12000 psi).

Soluble and membrane fractions were prepared by centrifugation at $23\,000 \times g$ for 30 min in the case of freeze-thawing or $100\,000 \times g$ for 60 min in the case of French pressing. Membranes were resuspended in 3–5 vols. of 10 mM sodium phosphate (pH 7).

Cytochromes *c*

Horse heart cytochrome *c* (Type VI) was from Sigma (Poole, U.K.). Cytochrome *c*-551 was prepared from *Pseudomonas stutzeri* (strain 224, ATCC 17591) by conventional methods.

Assay of peroxidase activity

Cytochrome-*c* peroxidase activity was measured by the decline in absorbance at the α -band of ferrocytochrome *c* (concentrations in Table legends) on addition of hydrogen peroxide (17 μ M) and a cell extract. The cytochrome *c* was reduced in situ in a stirred cuvette under argon by titration with 10 mM sodium dithionite (prepared anaerobically in 20 mM sodium phosphate (pH 7)). Anaerobic conditions were required to prevent interference by oxidase activity of small membrane vesicles, and also to minimise production of H₂O₂ from the reaction of dithionite with O₂. Such peroxide production led to variable re-oxidation of cytochrome *c* after addition of the cell supernatant, but prior to addition of H₂O₂.

The oxidation of the ferrocytochrome by peroxidase and hydrogen peroxide followed an exponential (pseudo-first-order) progress curve. Rate constants were derived from plots of $\log A_t - A_\infty$ against time (where A_t is the absorbance of the α -peak at time *t* and A_∞ is the absorbance after addition of ferricyanide). Velocities were derived from the initial reaction rate or were calculated from $v = k \times [C_r]$ where *k* = pseudo-first-order rate constant and *C_r* = concentration of ferrocytochrome *c*. The two methods gave similar results.

Assays of oxygen utilisation

Oxygen utilisation was measured in an oxygen electrode (Rank Brothers, Cambridge, U.K.) at 25°C in 10 mM sodium phosphate (pH 7)/1 mM EDTA, in a final volume of 2 ml. Substrates used were 1 mM ascorbate with 0.3 mM TMPD (Sigma) or 10 mM sodium formate. Experiments were also performed with pulses of 250 nmol formate.

The presence of hydrogen peroxide formed during these experiments could be detected by addition of 1400 Sigma units of catalase to release O₂ by dismutation.

HOQNO (2-heptyl-4-hydroxyquinoline-*N*-oxide, Sigma), when present, was preincubated for 2 min with membranes at a concentration of 50 μ M.

Assay of cytochrome-*c* oxidase activity

Cytochrome-*c* oxidase activity was measured by the decline in absorbance at the α -band of ferrocytochrome *c* on addition of a cell membrane fraction. The cytochrome *c* (9 μ M) was reduced in situ in a cuvette by the addition of sodium ascorbate (pH 7). Different final concentrations of ascorbate were required to produce comparable levels of reduction of the different cytochromes studied. 67 μ M ascorbate gave 96%, and 91% reduction in horse cytochrome *c*, and *Ps. stutzeri* cytochrome *c*-551, respectively, while 400 μ M ascorbate was required to produce 52% reduction in *C. mucosalis* cytochrome *c*-553. After a steady level of reduction had been achieved, addition of a cell membrane fraction resulted in oxidation of the cytochrome *c*, which followed an exponential progress curve to a new steady-state level of partial reduction. Velocities were derived from the initial rate of oxidation, monitored at the α -peak.

Proton translocation

The method used was that of Dawson and Jones [9]. The chamber contained 3 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma) (pH 7.2) 140 mM KCl, 200 μ g carbonic anhydrase, 20 μ g valinomycin (both from Sigma) and 0.1 ml fresh cell suspension (4 mg protein) in a final volume of 3.5 ml. The chamber was stirred, maintained at 27°C, and flushed with oxygen-free nitrogen.

Either formate (1 mM) or H₂ (0.78 mM) were present as substrates, the latter by flushing with H₂ gas in place of N₂. Pulses of O₂ (39 nmol) were added by injection of 0.15 ml of air-saturated buffer. Pulses of hydrogen peroxide (88 nmol) were injected in 10 μ l of O₂-free solution. 5 μ M CCCP (carbonyl cyanide *m*-chlorophenylhydrazide, Sigma) was present as required.

Acidification of the medium was recorded with a pH electrode connected to a chart recorder. The pH change was calibrated by addition of 0.01 M HCl or 0.01 M KOH and was found to be linear between pH 6.8 and 7.2.

Purification and properties of cytochrome *c*-553

All procedures were carried out at 4°C. The soluble cell extract from *C. mucosalis* (3 g wet weight cells) was passed through Sephadex G-25

(fine) equilibrated in 2 mM sodium phosphate (pH 7) and the coloured eluate was applied to a column (10 \times 2 cm) of Whatman DEAE-52 cellulose equilibrated in the same buffer. The unadsorbed eluate was applied to a column (8 \times 1 cm) of Whatman CM-52 cellulose and the adsorbed proteins were eluted with a linear gradient of 2–100 mM sodium phosphate (pH 7) (400 ml). Cytochrome *c*-553 was the only coloured protein eluted from this column, and red fractions were combined, diluted 6-fold reabsorbed to a small CM-52 cellulose column (1 \times 2 cm), and eluted as a sharp band in 100 mM sodium phosphate (pH 7). This solution was passed through a column of Sephadex G75 superfine equilibrated in 20 mM Tris-HCl (pH 8)/100 mM NaCl and the red fractions were diluted 10-fold, and concentrated on CM-52 as described above.

Amino-acid analysis of the purified cytochrome was carried out on a Locarte analyser after hydrolysis in 6 M HCl in vacuo for 70 h at 105°C. Cysteine was measured as cysteic acid after removal of the haem group [10], performic acid oxidation [11] and acid hydrolysis for 20 h. The sample for analysis contained 5.9 nmol haem, as determined by the pyridine haemochrome method. Tryptophan was not determined by amino-acid analysis. An estimate of the tryptophan content was obtained by subtracting the tyrosine and haem contributions at 280 nm from the absorbance of the holoprotein. This calculation assumes

$$A_{280\text{ nm}}^{\text{tyrosine}} = 1.1 \text{ mM}^{-1} \cdot \text{cm}^{-1} \text{ and } A_{280\text{ nm}}^{\text{haem}} = 13.8 \text{ mM}^{-1} \cdot \text{cm}^{-1}$$

the latter being calculated from cytochromes *c* of known tryptophan content.

Redox titration of cytochrome *c*-553 was carried out in 10 mM sodium phosphate (pH 7) in a stirred anaerobic cuvette fitted with a Pt:Ag|AgCl combination electrode (Russell pH, Auchtermuchty, U.K.). The redox buffer-mediator was 20 μ M ferric ammonium sulphate/400 μ M EDTA. Oxidative titration was performed with a potassium ferricyanide solution; reductive titration was performed with a ferrous ammonium sulphate solution.

Results

Respiratory models for cells after 1 day and 3 days growth

The relative importance of different pathways of oxygen utilisation by *C. mucosalis* changes during growth in a culture jar. The features of these different pathways are shown in Fig. 1 and the characterisation of the individual components of the model is described below.

Hydrogen peroxide production by *C. mucosalis*

Addition of small pulses of the respiratory substrate, formate, to whole cells resulted in O_2 uptake (Fig. 2). After exhaustion of formate, the presence of hydrogen peroxide can be detected by the addition of catalase, which releases O_2 by dismutation. More H_2O_2 was found in 'older' cells (Fig. 2a). However 'younger' cells accumulate

H_2O_2 if the quinone analogue and respiratory inhibitor HOQNO is present (Fig. 2c). Similar results (not shown) were obtained under conditions of formate excess when catalase was added during the process of oxygen uptake.

Because H_2O_2 accumulation is enhanced by HOQNO, we propose that its production occurs on the substrate side of the block. We have accordingly placed it in Fig. 1 as part of the formate-oxidising system.

After dismutation by catalase of any H_2O_2 present, the final level of oxygen uptake in Figs. 2a, b and c should be quantitatively related to the amount of formate added by the equation:



The measured stoichiometries ($HCOO^-/O_2$) for

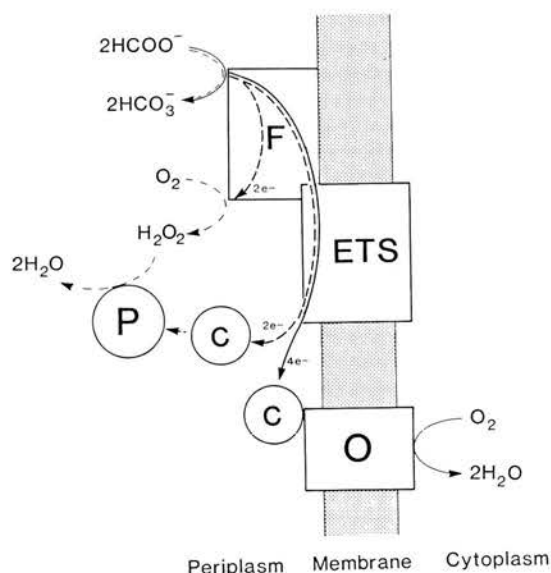


Fig. 1. Patterns of electron flow in the respiration of *C. mucosalis*. Two different pathways for the oxidation of formate by *C. mucosalis* are shown. In cells harvested early in growth, we propose that the broken line shows the dominant flow of electrons. This involves direct reduction of O_2 to hydrogen peroxide by a formate-oxidising enzyme (F) followed by reduction of hydrogen peroxide to H_2O by a cytochrome-*c* peroxidase (P) which receives electrons from the electron transport system (ETS) via a cytochrome *c*-553 (C). In cells harvested later in growth, the solid line shows the proposed dominant flow of electrons leading to a conventional reduction of O_2 to H_2O by a membrane-bound terminal oxidase (O).

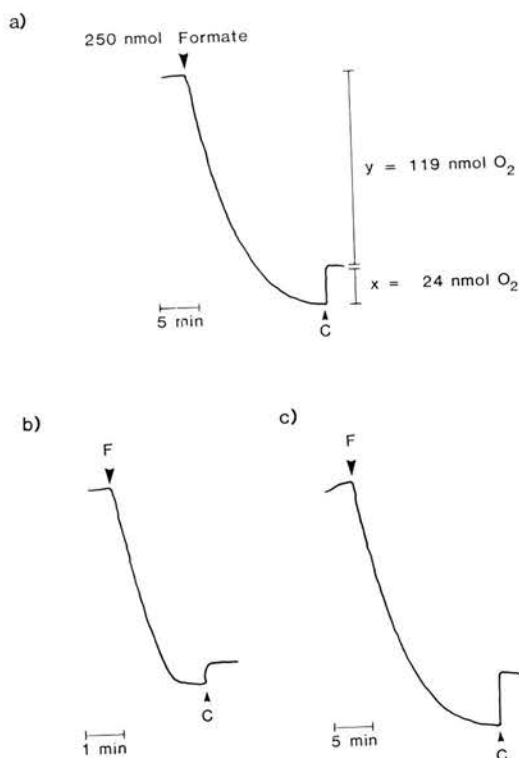


Fig. 2. The oxidation of formate by *C. mucosalis*. Formate pulses (250 nmol) were added to *C. mucosalis* grown for 3 days (a) or 1 day (b, c). HOQNO (50 μ M) was preincubated with the cells in (c). O_2 uptake was measured in an O_2 electrode (see Materials and Methods) and, after uptake was complete, catalase (C) was added.

Figs. 2a, b and c are 2.11, 2.22 and 2.06, respectively, in reasonable agreement with the expected figure of 2.

Prior to dismutation by catalase of any H_2O_2 present, the traces should enable calculation of the relative importance of partial reduction to H_2O_2 and complete reduction to H_2O [6]. For example, the value x/y in Fig. 2a gives the proportion of the flow of electrons from formate to give H_2O_2 ($= 20\%$). However, this is a useful estimate only if there is stable accumulation of H_2O_2 . In this organism, catalase is absent but removal of H_2O_2 may occur via the cytochrome-*c* peroxidase (see below). We propose that the enhanced accumulation of H_2O_2 in 'young' cells treated with HOQNO is due to blockage of the electron supply to the cytochrome-*c* peroxidase (Fig. 2c).

The properties of cytochrome c-553 and variation in amounts with age of cells

A soluble cytochrome *c*-type component with an asymmetric α -peak and a mid-point potential near $+100$ mV was identified by analysis of complex potentiometric titrations of soluble cell extracts from *C. mucosalis* [12]. This cytochrome has been purified by chromatography on CM-cellulose (see Materials and Methods) and its basic nature was confirmed by electrophoresis in the native state (results not shown).

On the basis of molecular exclusion chromatography on Sephadex G-75 and polyacrylamide gel electrophoresis in SDS (results not shown), cytochrome *c*-553 has a similar molecular weight to cytochrome *c*-551 of *Pseudomonas aeruginosa* (Molecular weight from amino-acid sequence, 9329 [13]). Parallel determinations of amino-acid composition and haem content showed that cytochrome *c*-553 contains 68–72 amino acids per haem group (Table I). In conjunction with the estimates of molecular weight, this indicates that the molecule has a single haem.

Spectroscopic properties of cytochrome *c*-553 are summarised in Table II with absorption coefficients based on pyridine haemochrome determination. The presence of a 695 nm band in the ferricytochrome suggests methionine coordination of the iron [14]. Potentiometric titration in the region of the α -band (Fig. 3a) was used to derive a mid-point potential ($E_{m,7}$) of $+99$ mV for a single

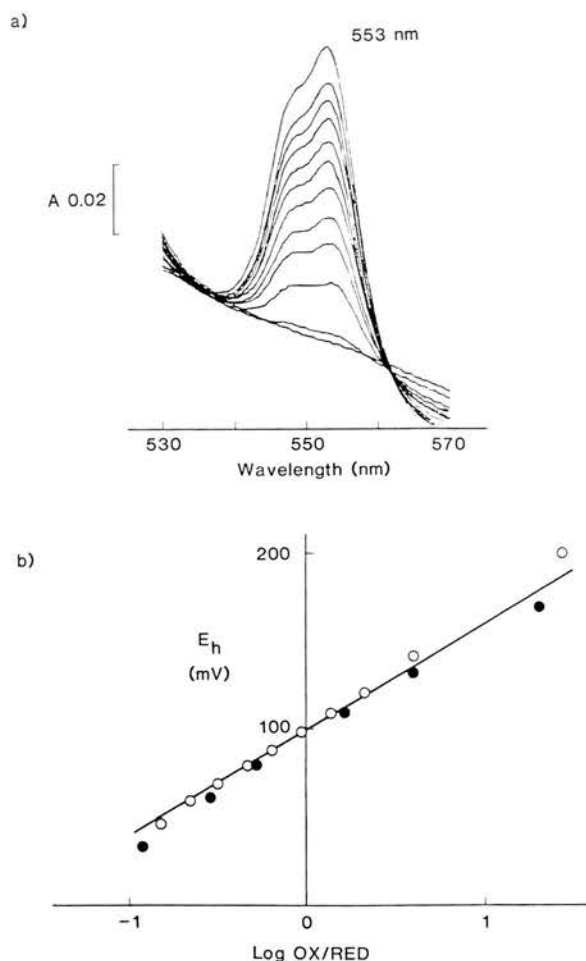


Fig. 3. Potentiometric redox titration of *C. mucosalis* cytochrome *c*-553. (a) Spectra were recorded during a reductive titration of cytochrome *c*-553 as described in materials and Methods. Each spectrum corresponds to a measured redox potential and was used to derive the plot shown in (b). (b) Points were calculated from the reductive titration (\circ) shown in (a), and from an oxidative titration (\bullet , not shown).

redox species (Fig. 3b) in good agreement with that predicted from titration of the cell extract [12].

2–5-times more cytochrome *c*-553 was present in cells grown for 1 day compared with those grown for 3 days. This was shown by molecular exclusion chromatography of the soluble extract, by quantitative analysis of haem-stained SDS electrophoretic gels and by the degree to which the α -band of a soluble extract was reducible with

TABLE I
AMINO-ACID COMPOSITION OF CYTOCHROME *c*-553

	nmol	mol/mol haem	Integer
Asp	70.2	11.9	12
Thr	19.5	3.3	3
Ser	7.1	1.2	1
Glu	10.2	1.7	2
Pro	22.5	3.8	4
Gly	31.8	5.4	5/6
Ala	65.2	11.1	11
Val	28.4	4.8	5
Met	14	2.4	2/3
Ile	24.3	4.1	4
Leu	20.2	3.4	3/4
Tyr	19.8	3.4	3/4
Phe	5.1	0.9	1
His	5.3	0.9	1
Lys	54.1	9.2	9
Arg	0	0	0
Cys		1.7	2
Trp		0	0
No of residues			68/72
M_r			7795–8259

ascorbate and phenazine methosulphate, since cytochrome *c*-553 is the only high potential cytochrome *c* present (results not shown).

The reactivity of cytochrome *c*-553 with cytochrome *c* peroxidase and cytochrome *c* oxidase of *C. mucosalis* is described below.

Cytochrome *c* peroxidase

Soluble extracts of *C. mucosalis* contain a cyto-

TABLE II
SPECTROSCOPIC PROPERTIES OF CYTOCHROME *c*-553

Figures in parentheses are absorption coefficients (mM^{-1} , cm^{-1}) based on pyridine haemochrome determination.

Ferricytochrome	Ferrocyclochrome
Spectral maxima	
280 (18.2)	Soret 416 (151.8)
Soret 408 (110.2)	β 522 (15.6)
524 (8.3)	α 553 (20)
695 (0.6)	
Spectral ratios	
α/β	1.28
$\alpha/280$	1.1

TABLE III
THE EFFECT OF CULTURE AGE ON OXIDASE AND PEROXIDASE ACTIVITIES

Age (days)	Cytochrome- <i>c</i> peroxidase ^a (nmol per min per mg cellular protein)	Ascorbate-TMPD oxidase ^b (nmol O ₂ per min per mg cellular protein)
1	230	119
3	60	210

^a The assay was as described in Materials and Methods, using horse ferrocyclochrome *c* ($8.5 \mu\text{M}$) in 10 mM phosphate (pH 7)/1 mM EDTA. Initial rates were calculated from $k \times [C_r]$, where k is the pseudo-first-order rate constant, and $[C_r]$ is the concentration of ferrocyclochrome *c*.

^b Rates were determined using the membrane fraction. The soluble fraction typically contained less than 10% of the oxidase activity.

chrome *c* peroxidase activity assayed using horse ferrocyclochrome *c* as substrate. This activity was nearly 4-times greater in cells grown for 1 day compared to 3-day cells (Table III). The peroxidase showed a preference for basic cytochromes (Table IV) and the cytochrome *c*-553 of *C. mucosalis* was the best donor tested under these particular conditions. Not only was the rate at $5 \mu\text{M}$ 3-times higher than that for horse cytochrome *c*, but the cytochrome *c*-553 approached saturation at this concentration, while horse cytochrome *c* was well below saturation. However, because of the small quantities of cytochrome *c*-553 available, routine assays were performed using horse ferrocyclochrome *c*.

Cytochrome-*c* oxidase

Almost all the ascorbate-TMPD oxidase activity of *C. mucosalis* was found in the membranes (Table III). The small amount of apparently soluble activity may be due to the presence of vesicles. Membranes from cells grown for 3 days contained twice the activity found in membranes from younger cells (Table III).

Ascorbate-TMPD oxidase activity is considered to reflect the presence of a cytochrome *c* donor to an oxidase [15]. Cytochrome-*c* oxidase activity itself was detected using ferrocyclochromes *c* as electron donors (Table IV). The assay is com-

TABLE IV

PEROXIDASE AND OXIDASE REACTIVITY WITH CYTOCHROME *c*

The oxidation of each cytochrome was monitored at the α -peak maximum.

	Net charge at pH 7	Cytochrome- <i>c</i> peroxidase ^a		Cytochrome- <i>c</i> oxidase ^d	
		<i>v</i> ($\mu\text{M} \cdot \text{min}^{-1}$)	%	<i>v</i> ($\mu\text{M} \cdot \text{min}^{-1}$)	%
<i>C. mucosalis</i> c-553	+ve	20.7 ^b	100	11.3 ^e	100
<i>C. mucosalis</i> c-553	+ve	16.3 ^c	79		
Horse cytochrome <i>c</i>	+ve	7.0 ^b	34	7.0 ^f	62
Horse cytochrome <i>c</i>	+ve	3.3 ^c	16		
<i>Ps. stutzeri</i> c-551	-ve	0.3 ^b	1	0.8 ^g	7

^a The assay was as described in Materials and Methods using: ^b 5.0 μM or ^c 3.3 μM ferrocytochrome *c* in 10 mM sodium phosphate (pH 7)/1 mM EDTA. The reaction was initiated using 25 μl soluble cell extract (derived from a broken cell suspension containing 20 mg protein per ml). Rates are expressed relative to 5 μM *C. mucosalis* c-553 (100%).

^d The assay was as described in Materials and Methods with 9 μM cytochrome *c* in 10 mM phosphate/1 mM EDTA. Cytochromes *c* were reduced with ascorbate. Concentration of ferrocytochrome *c* attained was: ^e 4.7 μM , ^f 8.6 μM or ^g 8.2 μM and the oxidation was initiated by 5 μl of a membrane fraction derived from a broken cell suspension containing 20 mg protein per ml. Rates are expressed relative to *C. mucosalis* c-553 (100%).

plicated by the tendency for cytochrome *c*-553 to oxidise in air. Also, unlike the peroxidase assay, the cytochrome cannot be reduced in the cuvette by dithionite because the latter reacts with the oxygen required for the oxidase reaction. Thus, the assay involves a reduction of the cytochrome in the cuvette using a low concentration of ascorbate. However, this, in itself, gives rise to two problems. One is that the subsequent oxidase rate is influenced by the excess ascorbate, which tends to re-reduce the cytochrome *c*. The contribution of this re-reduction rate increases as more oxidised cytochrome is formed until a steady-state level is reached. The second problem is that reduction is incomplete for some cytochromes (52% for cytochrome *c*-553), making it difficult to compare cytochromes under identical conditions. For these reasons, no attempt has been made to study the cytochrome concentration dependence of the reaction.

Nevertheless, the results of Table IV show the same clear qualitative preference for basic cytochromes as was seen for the peroxidase activity. Cytochrome *c*-553 was again more active than horse cytochrome *c*.

Topography of electron transport, energy conservation and hydrogen peroxide production

Our proposal in Fig. 1 incorporates a peri-

plasmic location for both H_2O_2 production by formate oxidation and H_2O_2 removal by cytochrome-*c* peroxidase action. Formation of spheroplasts and examination of the periplasmic and cytoplasmic fractions is an important method for establishing topography of bacterial respiratory processes. However, in spite of numerous attempts, we have been unable to form spheroplasts from *C. mucosalis*. In all cases, extensive damage to the cell membrane and release of isocitrate dehydrogenase or malate dehydrogenase as cytoplasmic marker enzymes was observed. However, although we have no such direct evidence for the topography outlined in Fig. 1, persuasive arguments can be offered in its favour.

Thus, it is probable that the cytochrome-*c* peroxidase is periplasmic. Wood [16] has proposed what may be a general rule, that *c*-type cytochromes are either periplasmic or located at the periplasmic side of the cell membrane. We have proposed that the substrate of the peroxidase is cytochrome *c*-553, suggesting a periplasmic location for both.

We propose that hydrogen peroxide production is also periplasmic. Thus, if formate oxidase is facing the periplasmic side of the cell membrane, the pattern of H^+ release and uptake during formate oxidation with H_2O_2 as an intermediate is shown in Fig. 4. In this diagram, there is no net

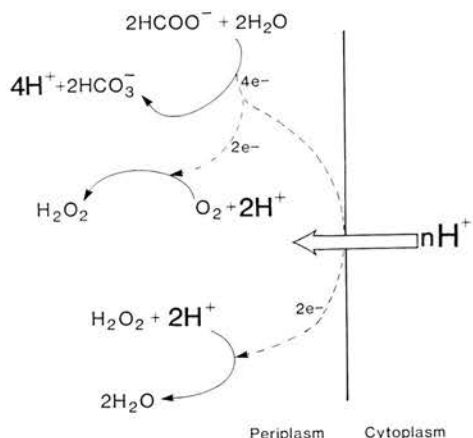


Fig. 4. A model for energy conservation during oxidation of formate by *C. mucosalis*. The diagram is a modified form of that shown in Fig. 1, in which only the electron flow predominating after 1 day of growth is considered. The membrane separating the periplasm and cytoplasm is shown as a single vertical line. Proton release and uptake in the periplasmic space are emphasised, and balance. Protons translocated across the membrane to acidify the periplasm are shown by the open arrow. Under anaerobic conditions, with a pulse of H_2O_2 as electron acceptor (as in Fig. 5), there is no reduction of oxygen and all four electrons from the two formate molecules will pass via the electron transport system to reduce two molecules of H_2O_2 . This again results in a balance of proton release and uptake in the periplasmic space.

change in the H^+ concentration in the periplasmic space due to the reactions themselves, while if formate oxidase were cytoplasmic, there would be an acidification inside the cell and an alkalisation outside. Thus, the only possible contribution to a bioenergetically useful acidification of the periplasm would have to come from the protons pumped by the electron transport system (shown as the transmembrane arrow in Fig. 4).

Whole cells generated a proton gradient, as measured by acidification of the external medium with either formate or hydrogen as substrates and pulses of oxygen as electron acceptor. Similar acidification was observed with H_2O_2 as oxidant. In both cases, the effects were abolished by the protonophore CCCP (Fig. 5).

The stoichiometry of H^+ pumping is very low for both oxygen (approx. $0.7 \text{ H}^+/\text{O}$) and H_2O_2 (approx. $0.6 \text{ H}^+/\text{H}_2\text{O}_2$). It seems probable that this reflects the fragility of the cell membrane of *C. mucosalis* already seen in attempts to form spheroplasts. Thus, in the proton extrusion experi-

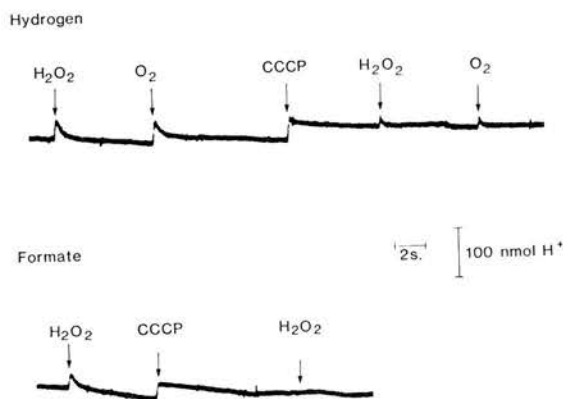


Fig. 5. Proton effects during respiration of *C. mucosalis*. The acidification of the medium after addition of pulses of O_2 or hydrogen peroxide to suspensions of *C. mucosalis* was measured as described in Materials and Methods. Acidification was studied in the presence of two substrates, hydrogen (top trace) and formate (lower trace).

ment, only a small proportion of cells may have an intact membrane and therefore be capable of producing a vectorial H^+ effect.

From these results, it is probable that the mechanism for removal of H_2O_2 may act not only to detoxify this compound, but may also contribute to energy conservation via a proton-pumping electron transport system. In that case, the bacteriological status of *C. mucosalis* as catalase-negative is interesting because the presence of catalase would remove this possibility of energy conservation, and reduce growth. We have tested this directly by comparing the growth of *C. mucosalis* on plates containing blood (and therefore erythrocyte catalase) with plates containing serum. The qualitative observation was that cells grew faster on plates lacking catalase.

Discussion

The sealed culture jars used for the growth of *C. mucosalis* initially contain oxygen at 20% of atmospheric level, which then decreases due to cellular respiration. We propose that this change in oxygen concentration is accompanied by a change in the nature of the respiration. When the oxygen concentration is relatively high in the early stages of growth, the oxidation of formate involves the production of hydrogen peroxide. This is then

reduced to water by a cytochrome-*c* peroxidase which receives electrons from the membrane electron transport system via a cytochrome *c*-553. Thus, this pattern of respiration involves a two-stage reduction of oxygen to water and a branched flow of electrons (Fig. 1). As the oxygen concentration falls in the culture jar, less hydrogen peroxide is formed and the electron flow occurs predominantly by a conventional linear pathway to a terminal cytochrome-*c* oxidase.

We examined the changes that occur in individual components of this model during adaptation to lower oxygen concentrations. Although little accumulation of hydrogen peroxide by cells harvested early in growth could be detected, due to its rapid removal by the peroxidase pathway, the capacity for large-scale production of hydrogen peroxide could be seen in cells inhibited by HOQNO (Fig. 2c). The rapid removal of the hydrogen peroxide in early growth was achieved by elevated levels of cytochrome-*c* peroxidase and its specific electron donor, cytochrome *c*-553. In later stages of growth, lower levels of these components, but higher levels of cytochrome oxidase, were observed. Although the oxidase also utilises cytochrome *c*-553, less of this may be required to efficiently mediate electron transfer between membrane complexes than between a membrane complex and a soluble peroxidase.

This adaptive response of *C. mucosalis* to changing oxygen concentrations is relevant to our understanding of microaerophilic bacteria. One possible reason for the toxicity of oxygen to such bacteria is the presence of autoxidisable dehydrogenases which generate hydrogen peroxide by direct interaction with oxygen [1]. Formate dehydrogenase may be one such enzyme in *C. sputorum* [4,5], *C. jejuni* [17] and *C. mucosalis*. If such enzymes have a relatively high K_m for oxygen [4], the rate of production of the toxic product will increase as the oxygen concentration is raised. With *C. mucosalis*, we propose that induction of the peroxidase system allows some adaptation, but a point will be reached where the removal of the hydrogen peroxide cannot keep pace with its production, and cell death occurs.

The status of catalase in this scheme is of interest. Both *C. mucosalis* and *C. sputorum* are catalase-negative but, while the addition of cata-

lase to the medium can enhance the aerotolerance of some microaerophiles, this is not the case for these two organisms. A possible explanation for this is that, although catalase will allow removal of the toxic hydrogen peroxide, it will also eliminate the possibility of energy conservation by a proton-translocating electron transport chain that supplies electrons to a peroxidase. We have demonstrated that such proton translocation can occur in response to pulses of hydrogen peroxide in *C. mucosalis*. Thus, although production of toxic hydrogen peroxide seems unavoidable in this organism, once formed, it appears to play a positive role in energy conservation. This novel method of energy conservation may not be available to catalase-positive organisms, or to parasitic organisms that are exposed to the peroxide-scavenging mechanisms of their hosts.

Another possible reason for the failure of catalase to enhance oxygen tolerance in *C. mucosalis* is one of access. If hydrogen peroxide causes damage close to its site of production in the cell, the presence of added catalase in the medium may be ineffective. In this connection, it is of interest to note that some bacteria may possess both catalase and peroxidase. This apparent duplication of function might be explained if catalase were cytoplasmic and dealt with hydrogen peroxide generated in that compartment, while cytochrome-*c* peroxidase removed periplasmic hydrogen peroxide. Such locations for catalase and cytochrome-*c* peroxidase have been demonstrated in *Ps. stutzeri* (Wilson, Goodhew, Hunter and Pettigrew, unpublished data).

A common pattern in biological electron transport is for a small, soluble, high potential cytochrome *c* (Class 1 of Ambler [18]) to act as donor to both a membrane oxidase and soluble enzymes [19]. In size, mid-point potential and spectrum, the cytochrome *c*-553 of *C. mucosalis* clearly falls within Class 1, although it is in a small minority of basic cytochromes. Other *Campylobacter* species probably contain related high-potential cytochromes *c* [5,20,21]. Of the Class 1 cytochromes, cytochrome *c*-553 most closely resembles cytochrome *c*-555 from *Chlorobium limicola* f. *thiosulphatophilum* in spectrum, basic nature and mid-point redox potential. Indeed, we have shown that this cytochrome *c*-555 was reactive with both the per-

oxidase and oxidase of *C. mucosalis* (results not shown), although this apparent similarity is somewhat surprising in view of the disparate lifestyles of the two organisms. However, precedents exist for the occurrence of similar cytochromes in apparently divergent bacterial genera [18].

In conclusion, we have presented evidence of the production of H_2O_2 by *C. mucosalis*, and the utilisation of H_2O_2 and O_2 as terminal acceptors for energy-conserving electron transfer through the respiratory chain. The reactivity of *c*-553 with the peroxidase and oxidase, and the levels of these proteins during growth, was consistent with the proposal that cytochrome *c*-553 donates electrons predominantly to the peroxidase in the early stages of growth, and subsequently to the oxidase in later growth.

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